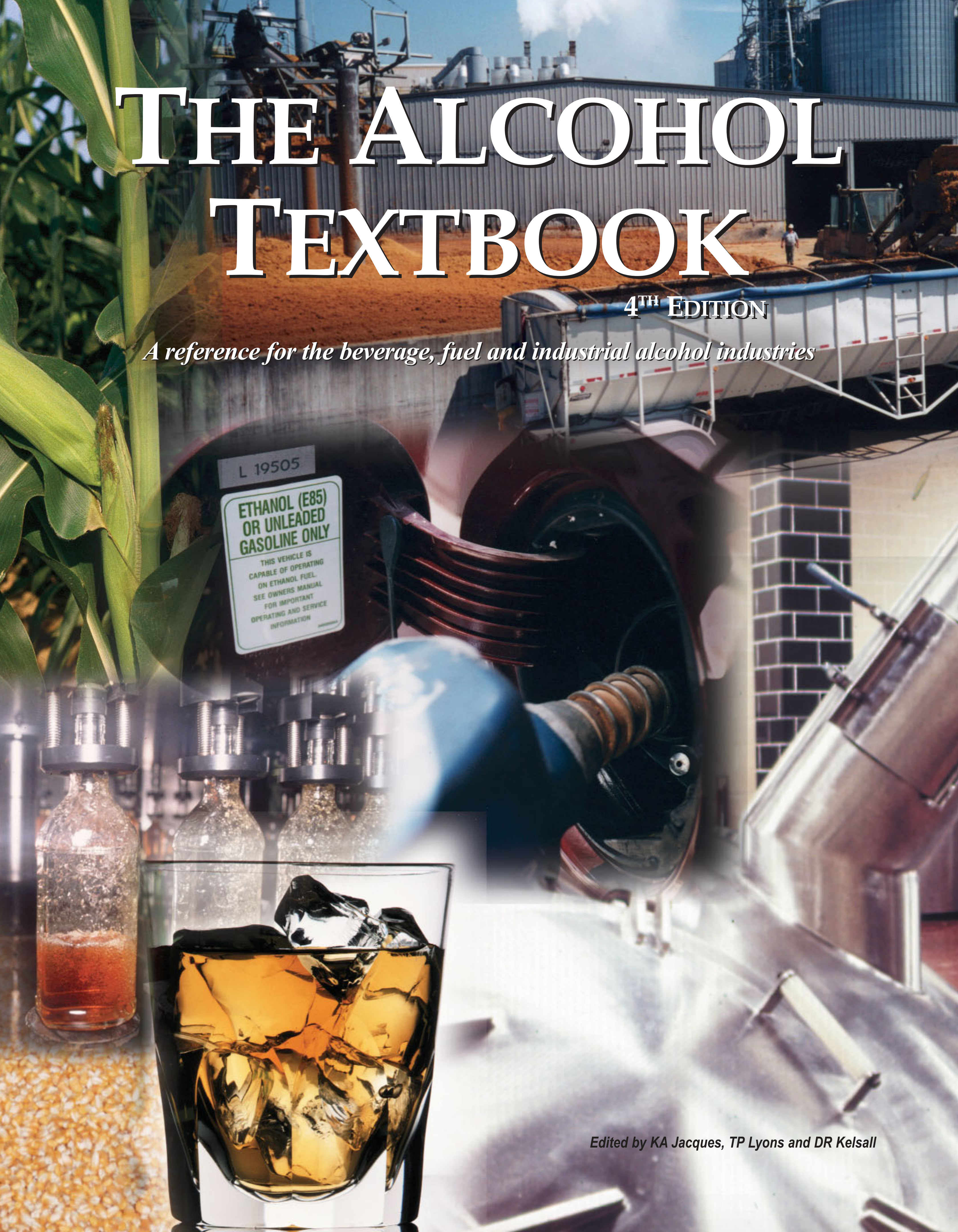
THE ALCOHOL TEXTBOOK

4TH EDITION

A reference for the beverage, fuel and industrial alcohol industries


L 19505
ETHANOL (E85)
OR UNLEADED
GASOLINE ONLY
THIS VEHICLE IS
CAPABLE OF OPERATING
ON ETHANOL FUEL.
SEE OWNERS MANUAL
FOR IMPORTANT
OPERATING AND SERVICE
INFORMATION

Edited by KA Jacques, TP Lyons and DR Kelsall

The Alcohol Textbook

4th Edition

A reference for the beverage, fuel and industrial alcohol industries

K.A. Jacques, PhD
T.P. Lyons, PhD
D.R. Kelsall

Nottingham University Press
Manor Farm, Main Street, Thrumpton
Nottingham, NG11 0AX, United Kingdom

NOTTINGHAM

Published by Nottingham University Press (2nd Edition) 1995
Third edition published 1999
Fourth edition published 2003
© Alltech Inc 2003

All rights reserved. No part of this publication may be reproduced in any material form (including photocopying or storing in any medium by electronic means and whether or not transiently or incidentally to some other use of this publication) without the written permission of the copyright holder except in accordance with the provisions of the Copyright, Designs and Patents Act 1988. Applications for the copyright holder's written permission to reproduce any part of this publication should be addressed to the publishers.

ISBN 1-897676-13-1

Page layout and design by Nottingham University Press, Nottingham
Printed and bound by Bath Press, Bath, England

Contents

Foreword ix
T. Pearse Lyons
Presient, Alltech Inc., Nicholasville, Kentucky, USA

Ethanol industry today

1 Ethanol around the world: rapid growth in policies, technology and production 1
T. Pearse Lyons
Alltech Inc., Nicholasville, Kentucky, USA

Raw material handling and processing

2 Grain dry milling and cooking procedures: extracting sugars in preparation for fermentation 9
Dave R. Kelsall and T. Pearse Lyons
Alltech Inc., Nicholasville, Kentucky, USA

3 Enzymatic conversion of starch to fermentable sugars 23
Ronan F. Power
North American Biosciences Center, Alltech Inc., Nicholasville, Kentucky, USA

4 Grain handling: a critical aspect of distillery operation 33
David J. Radzanowski
Alltech Inc., Nicholasville, Kentucky, USA

Substrates for ethanol production

5 Lignocellulosics to ethanol: meeting ethanol demand in the future 41
Charles A. Abbas
Archer Daniels Midland, Decatur, Illinois, USA

6 Ethanol production from cassava 59
Nguyen T.T. Vinh
The Research Institute of Brewing, Hanoi, Vietnam

7 Whey alcohol - a viable outlet for whey? 65
Jack O'Shea
Alltech Inc., Dunboyne, County Meath, Ireland

8 Treatment and fermentation of molasses when making rum-type spirits 75
Robert Piggot
Alltech Inc., Nicholasville, Kentucky, USA

Yeast and management of fermentation

9 **Understanding yeast fundamentals** 85
Inge Russell
International Centre for Brewing and Distilling, School of Life Sciences, Heriot-Watt University, Edinburgh, UK

10 **Practical management of yeast: conversion of sugars to ethanol** 121
Dave R. Kelsall and T. Pearse Lyons
Alltech Inc., Nicholasville, Kentucky, USA

11 **Continuous fermentation in the fuel alcohol industry: How does the technology affect yeast?** 135
W. M. Ingledew
University of Saskatchewan, Saskatoon, Saskatchewan, Canada

12 **Understanding near infrared spectroscopy and its applications in the distillery** 145
Don Livermore[1], Qian Wang[2] and Richard S. Jackson[2]
[1]Hiram Walker & Sons Ltd., Walkerville, Ontario, Canada
[2]Bruker Optics Inc., Billerica, Massachusetts, USA

13 **Emerging biorefineries and biotechnological applications of nonconventional yeast: now and in the future** 171
Charles A. Abbas
Archer Daniels Midland, Decatur, Illinois, USA

Beverage alcohol production

14 **Production of Scotch and Irish whiskies: their history and evolution** 193
T. Pearse Lyons
Alltech Inc., Nicholasville, Kentucky, USA

15 **Tequila production from agave: historical influences and contemporary processes** 223
Miguel Cedeño Cruz
Tequila Herradura, S.A. de C.V. Ex-Hda San Jose del Refugio Amatitán, Jalisco, México

16 **Production of heavy and light rums: fermentation and maturation** 247
Robert Piggot
Alltech Inc., Nicholasville, Kentucky, USA

17 **From pot stills to continuous stills: flavor modification by distillation** 255
Robert Piggot
Alltech Inc., Nicholasville, Kentucky, USA

18 **From liqueurs to 'malternatives': the art of flavoring and compounding alcohol** 265
Andy Head and Becky Timmons
North American Biosciences Center, Alltech Inc., Nicholasville, Kentucky, USA

19 **Production of American whiskies: bourbon, corn, rye and Tennessee** 275
Ron Ralph
Ron Ralph & Associates Inc., Louisville, Kentucky, USA

Contamination and hygiene

20 **Bacterial contamination and control in ethanol production** 287
N.V. Narendranath
North American Biosciences Center, Alltech Inc., Nicholasville, Kentucky, USA

21 **Managing the four Ts of cleaning and sanitizing: time, temperature, titration and turbulence** 299
Jim Larson and Joe Power
North American Biosciences Center, Alltech Inc., Nicholasville, Kentucky, USA

Recovery

22 **Ethanol distillation: the fundamentals** 319
P. W. Madson
KATZEN International, Inc., Cincinnati, Ohio, USA

23 **Development and operation of the molecular sieve: an industry standard** 337
R. L. Bibb Swain
Delta-T Corporation, Williamsburg, Virginia, USA

Engineering ethanol fermentations

24 **Water reuse in fuel alcohol plants: effect on fermentation**
Is a ‘zero discharge’ concept attainable? 343
W. M. Ingledew
University of Saskatchewan, Saskatoon, Saskatchewan, Canada

25 **Understanding energy use and energy users in contemporary ethanol plants** 355
John Meredith
Ro-Tech, Inc., Louisville, Kentucky, USA

The dryhouse, co-products and the future

26 **Dryhouse design: focusing on reliability and return on investment** 363
John Meredith
Ro-Tech, Inc., Louisville, Kentucky, USA

27 **Ethanol production and the modern livestock feed industry: a relationship continuing to grow** 377
Kate A. Jacques
North American Biosciences Center, Alltech Inc., Nicholasville, Kentucky, USA

28 **Biorefineries: the versatile fermentation plants of the future** 387
Karl A. Dawson
North American Biosciences Center, Alltech Inc., Nicholasville, Kentucky, USA

The Alcohol Alphabet 399
A glossary of terms used in the ethanol-producing industries

Index 441

Foreword

Alcohol production: a traditional process changing rapidly

T. PEARSE LYONS

President, Alltech Inc., Nicholasville, Kentucky, USA

In its simplest form, alcohol production is the process of preparing starch- or sugar-containing raw materials for fermentation by yeast, which is currently the only microorganism used for converting sugar into alcohol. The ethanol is then concentrated and recovered in a process called distillation. Though essentially a simple process, making it happen with maximum efficiency on a very large scale is a remarkable combination of microbiology and engineering.

Over the past 25 years progress has been made in virtually all aspects of the process, but what are the challenges facing alcohol in the future? Beginning with raw material processing, here are the areas that we feel need to be addressed:

Raw material reception and processing

Despite many declarations to the contrary, it is virtually impossible for a distillery to accurately calculate true yields. A sampling of grains is either done in very cursory fashion or not at all. The distiller must see himself first as a processor of grain rather than a producer of alcohol. He must know accurately how much starch is present in his raw material and he must be able to measure that immediately. NIR spectroscopy has much to offer in terms of raw material applications; and this topic is detailed in this volume. However, if we control the process and use the appropriate enzymes from solid state fermentation, such as Rhizozyme™ and Allzyme™ SSF, which are capable of releasing not just bound starch but also hemicellulose and cellulose, we can and indeed some plants do, get 2.9+ gallons per bushel (116 gallons or 420 liters/tonne. In fact, we predict yields of 3.2 gallons in the near future. For molasses, the advent of detranases can ensure maximum yields. Every aspect of cooking and fermentation must be controlled, however.

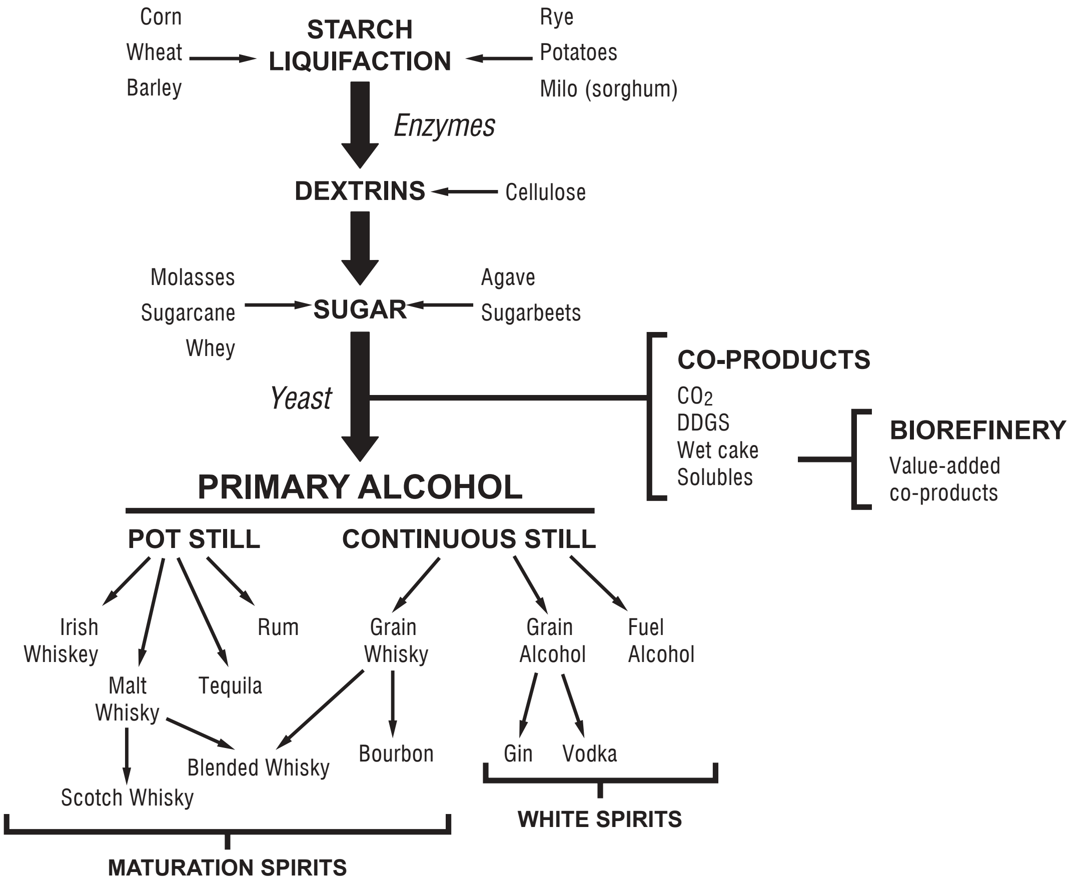
The cooking process

Much debate exists at the moment regarding the cooking process. It is a sad reflection perhaps on our industry that since the pioneering PhD work by Dr. John Murtagh in 1972 no further scientific thesis has been published on cooking. Should grain be finely ground? Should cooking involve high temperature α-amylase or should an entirely different type of enzyme be used? Often the enzyme selected is one developed based on experience in the corn wet milling sector, which may be far from ideal for dry mill alcohol production.

Our industry needs a new enzyme, one tailored to our specific needs: converting grain to fermentable sugar. Raw materials contain not only starch, but also hemicellulose (a polymer of 5-carbon sugars) and cellulose. The cooking process must be designed to loosen these materials such that they release bound starch and also become available in turn for fermentation.

Yeast and fermentation

Despite all we know about it, yeast remains underrated and misunderstood. It is the workhorse of the distillery, yet ‘economies’ on quality and quantity of yeast are made; and we


Corn
Wheat
Barley
STARCH LIQUIFACTION
Rye
Potatoes
Milo (sorghum)
Enzymes
DEXTRINS
Cellulose
Molasses
Sugarcane
Whey
SUGAR
Agave
Sugarbeets
Yeast
CO-PRODUCTS
CO2
DDGS
Wet cake
Solubles
BIOREFINERY
Value-added co-products
PRIMARY ALCOHOL
POT STILL
CONTINUOUS STILL
Irish Whiskey
Rum
Malt Whisky
Tequila
Grain Whisky
Grain Alcohol
Fuel Alcohol
Blended Whisky
Bourbon
Gin
Vodka
Scotch Whisky
WHITE SPIRITS
MATURATION SPIRITS

often force it to grow and operate under unsuitable conditions. As Professor Mike Ingledew has pointed out many times, *the fermentor is not a garbage can*! We must make sure that conditions in fermentation are optimum for yeast.

Like our need for appropriate enzymes, ethanol production also requires yeast with higher ethanol and temperature tolerance if we are to keep progressing to higher levels of alcohol in the fermentor. Today, 17-20% abv is becoming standard in our industry as we move to high temperature Thermosacc™-like yeast. When used in combination with enzymes capable of extracting increasing amounts of sugar, such as those produced by surface culture fermentation, yeast can bring us to a whole new level.

While 23% ethanol by volume in the fermentor without loss of performance is possible given the right yeast and enzyme combination, it can only be done by careful management of the so-called stress factors. Factors which, if left uncontrolled, will prevent the yeast from performing. Operating a fermentation at 23% ethanol is totally different than operating a distillery at 8% ethanol. Any minor change, whether in mycotoxin level, salt level or nutrient level can have a major impact on performance.

Microbial contamination

A distillery is never going to be a sterile fermentation and even those of us who have had the good fortune to operate in sterile fermentation conditions know how easily infection can take hold. Since the yeast is a relatively slow-growing microorganism compared to most of the infectious microorganisms (*Lactobacillus*, etc.), it is critical that we have an arsenal of antimicrobial agents and effective cleaning products and programs ready to protect the fermentor environment.

Recovery and utilization of co-products

As we review our overall process, it is obviously critical that we maximize utilization of co-products. Assuming the feedstock is corn, the major cost factors are the raw material, energy, enzymes, processing chemical cost (water treatment, etc.) and labor. Much of these costs can be offset with 17 lbs of distillers grains coming from each bushel of corn; but we must maximize the return on this essential product. Distillers grains with solubles (DDGS) are currently (July 2003) selling for around $80/ton and corn for $2.20 per bushel. At $0.04 per lb ($80/ton), 17 lbs of DDGS gives us a credit of $0.68. This is set against the $2.20 bushel of corn or the $0.03-0.04 for enzymes. If on the other hand, new or novel products could be made from distillers grains, (i.e. improved by-pass proteins for cattle, pre-hydrolyzed DDGS for human foods), the financial impact could be substantial. The future will see many new products built around DDGS. This is the subject of a separate chapter in this volume.

As dry mills shift toward biorefining, instead of simply grinding, corn, yield of ethanol becomes only one part of the economic equation. Perhaps in the future, further processing of distillers grains will create products that make spent grains the single most important raw material from a distillery. After all, they are rich and valuable protein sources, a commodity in very short supply across the world.

Education

Perhaps the biggest surprise, as one surveys the progress that has been made in the last 20 years is how little progress has been made in the area of education. Unlike the brewing industry, which seems to have an abundance of professionals with PhD and masters degrees, our industry has very few. Unlike the brewing industry, which has no less than five brewing schools around the world, the annual Alltech Alcohol School remains the only venue for industry-wide training.

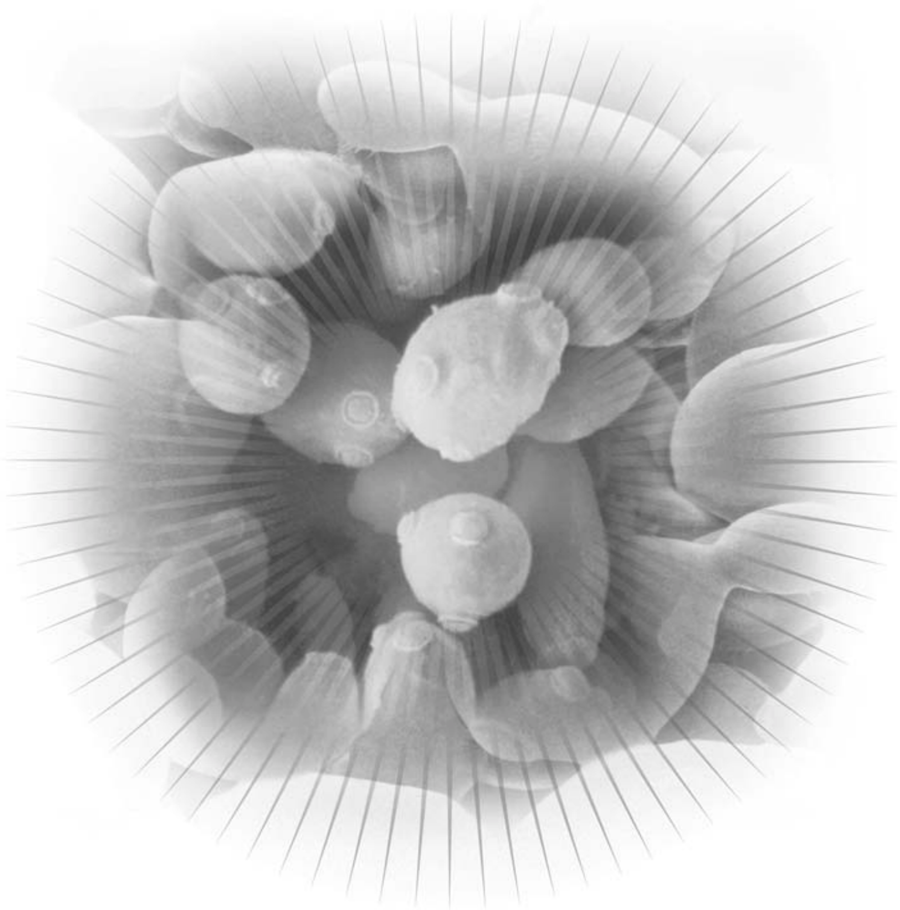
The Alltech Institute of Brewing and Distilling, established in 2000, set out to address this shortfall by working in conjunction with the Heriot-Watt University in Scotland. Perhaps our industry should use the brewing industry as a model. Scholarships should be funded and perhaps a bioscience center concept established. Bioscience centers bridge the gap between university and industry by creating an environment where students are encouraged to complete higher degrees while doing industry-focused research.

Conclusions

The alcohol industry, therefore, is alive and well, and we have been given an opportunity to achieve something that many of us thought would have been achieved by the mid-1980s. We have a superb oxygenate that will help reduce global warming and at the same time enable us to add value to our grains. The world is not short of starch and sugar, the world is short of protein. The fuel alcohol industry and beverage alcohol industries therefore are in fact generators of protein; it only remains for us to make sure the proteins we generate are the most advantageous possible to man and beast.

The chapters in this book we hope will help the reader realize the complexity and at the same time the simplicity of the process of converting sugars to ethanol. If we make your search for information just a little easier, we will have achieved our objective. If we can encourage more research, then we will be well-rewarded.

Ethanol industry today



Chapter 1

Ethanol around the world: rapid growth in policies, technology and production

T. Pearse Lyons

President, Alltech Inc., Nicholasville, Kentucky, USA

Fuel ethanol industry history: how did today's ethanol 'boom' happen?

The amazing expansion of the past few years is happening in a wholly different industry than when the first 'boom' occurred. In the late 1970s and early 1980s an embryonic ethanol industry was heralded as the renewable answer to worldwide shortages of fossil fuels. Farmers in America's heartland had abundant raw materials in the form of cereal grain; and it seemed at one stage as if every farm would have its own 'gasohol' plant. Small-scale operations with a myriad of new technologies emerged with unique names like 'OPEC Killer' or 'A Step to Independence'.

Few of the early fuel ethanol plants were really big, as it seemed the beverage plant size was taken as an example. Many were only capable of producing hundreds of gallons per day, but others were well-engineered plants with capacities up to 15-20 million liters per annum. Competitions were held at state fairs in order to enable farmers to determine which design was best. Enthusiasm, regardless of expertise, was the order of the day. The industry was crying out for training and technology; and during this time Alltech Inc. emerged, with its philosophy of marketing through education. Here also emerged the concept of the 'Alcohol Textbook', and the annual Alcohol School, which is now in its 23rd year.

ALLTECH
presents
GASOHOL
A Step to Energy Independence
A Step to Energy Independence
a textbook for fuel
by T.P. Lyons
The Alcohol Textbook
The Alcohol Textbook
The Alcohol Textbook
Third Edition
A reference for the beverage, fuel and industrial alcohol industries
The Alcohol Textbook Third Edition
PRIMARY ALCOHOL

Figure 1. The alcohol industry reference books by Alltech Inc.

By the mid-1980s, the smaller plants were beginning to run into typical start-up problems, many associated with poor design, little capital, and relatively high labor costs. Perhaps they were doomed to fail from the beginning, with or without government support. Production was nowhere near the forecasted 7 billion liters (1.85 billion gallons), and instead languished around 1.5 billion liters (0.4 billion gallons). Beverage ethanol remained the dominant ethanol product. To most, it looked like the gasohol boom was over.

While smaller operations disappeared, other larger companies, well funded and with well-designed plants, continued to grow. Corporations such as Cargill, Staleys and ADM became industry leaders. Today, one of these (ADM) is responsible for processing 45% of the corn that is converted into ethanol in the US. This company and others of this size knew the ethanol industry had long term prospects and they planned survival strategies accordingly.

Meanwhile in Brazil during the 1970s and 1980s, a similar revolution was occurring. In 1975 the so-called 'Proalcohol' program was launched, with massive support by the government. Initially, the program focused on the production of anhydrous ethanol, which was blended with gasoline to produce the Brazilian gasohol. The emphasis quickly changed to hydrous ethanol (95% ethanol/5% water), which could be used in its pure form in cars with specially designed engines. By the mid-1980s, such cars represented the vast majority of the marketplace and it seemed the Proalcohol product was going to replace gasoline on a 1:1 basis.

The system, however, was very inflexible. When ethanol became difficult to get in the late 1980s, consumers began to switch back to conventional cars in which gasoline (still blended with a minimum level of ethanol) could be used. Was the fuel ethanol program also going to falter in Brazil?

Growing interest in fuel ethanol around the world

Against such a background of declining interest in ethanol in the early 1990s, what brought about the resurrection of the ethanol boom in the US and elsewhere? The first driving factor was an international agreement called the Kyoto Protocol, in which most industrial nations agreed to improve the environment and lessen the greenhouse effect by burning less fossil fuel or doing so more efficiently. It was agreed to roll back carbon dioxide emissions to a given year. The impact was to be enormous.

UNPRECEDENTED GROWTH IN THE US

In the United States, domestic events have given the industry a big push. The US Clean Air Act mandated an average of 2% oxygen be present in what was called 'reformulated gasoline' (RFG). Representing some 30% of the vast US gasoline market (100 billion gallons), it created a clear demand for an oxygenate. MTBE (methyl tertiary butyl ether), with approximately 18% oxygen, and ethanol with 30% oxygen were chosen. Both were excellent oxygen boosters. MTBE, a petroleum derivative, was the early choice, but a series of scares regarding its possible carcinogenic nature and its detection in groundwater led to its planned phaseout. The alcohol industry, meanwhile, was beginning to emerge, particularly in the Corn Belt (Nebraska, Iowa, Minnesota and Illinois). Its capacity had reached 10 billion liters (2.7 billion gallons) by 2003 (Figure 2) and in anticipation of the phaseout of MTBE (not just in California, but around the country) some 73 plants had been built. At the time we are going to press, these 73 plants, located in 20 states, have the capacity of 11 billion liters (Figure 3). A further 13 plants are under construction, which will add some 2 billion liters (500 million gallons) of capacity.

The ethanol industry also continues its streak of monthly production records. An all-time monthly production record of 181,000 barrels per day (b/d) was set in June of 2003, according to the US Energy Information Administration (Figure 4). The previous all-time record was 179,000 b/d, set in April of this year.

Driving industry growth: Farmer-owned ethanol plants

Unlike the earlier plants, which were dominated by the large corporations, most of the new plants are farmer-owned projects, as farmers seek to add value to the commodities they grow and participate in profit sharing when the plant prospers. These plants have a typical size of 150 million liters and a construction cost per liter of $0.40-$0.50. The predominant raw material is locally-grown corn (maize); and ethanol production is the third largest and fastest growing market for US corn (Figure 5). In 2002, over 800 million bushels of corn were processed into ethanol and valuable feed co-products. The US Department of Agriculture estimates that more than one billion bushels of corn will be

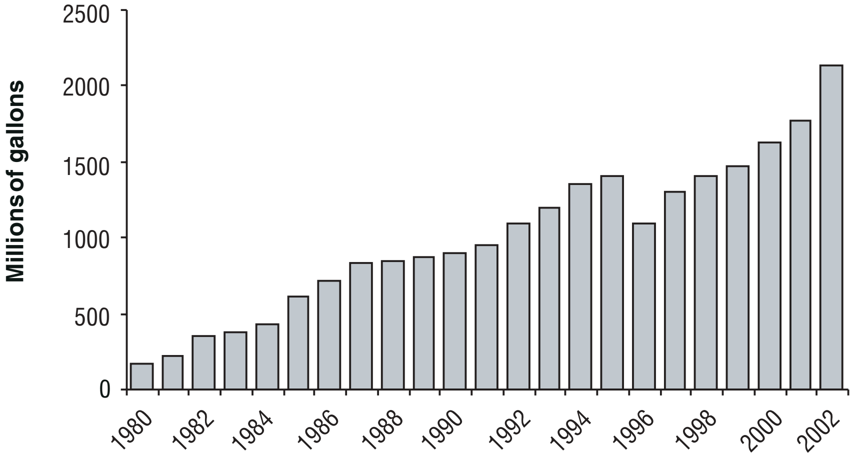
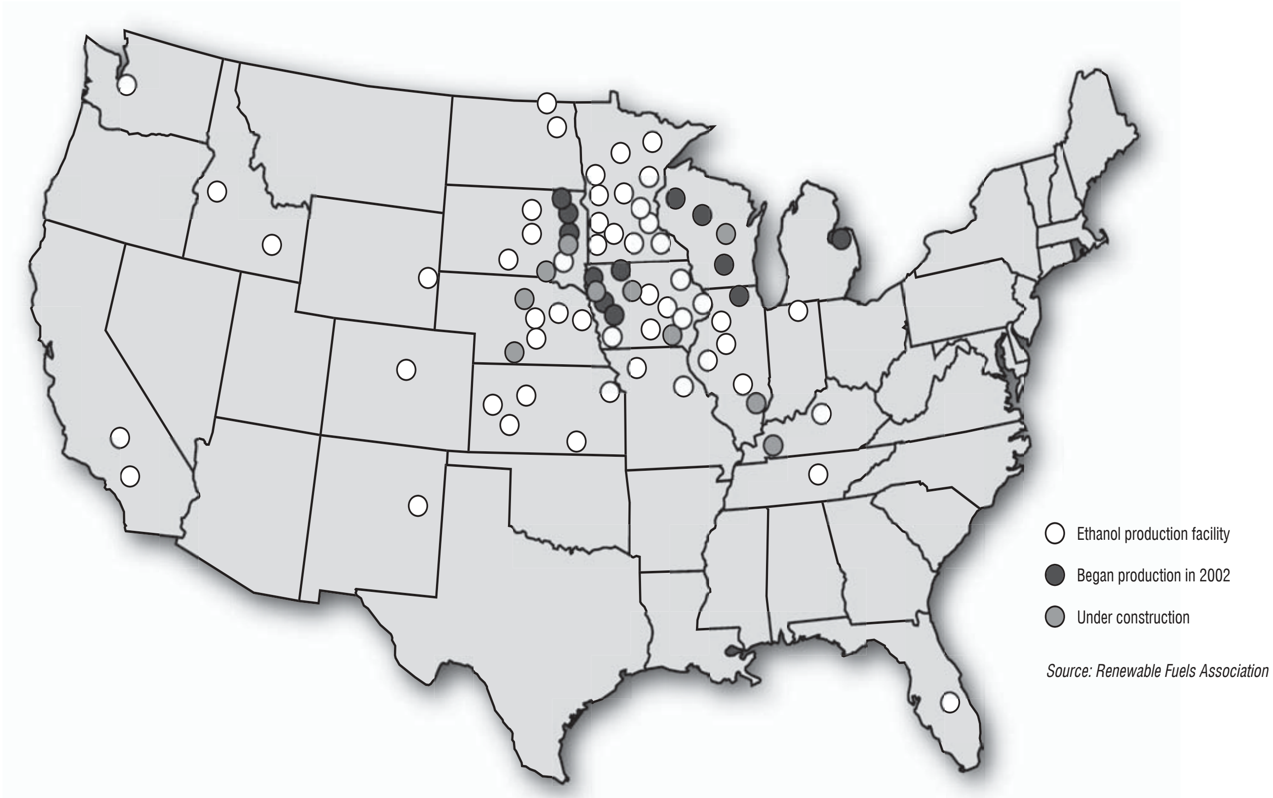


Figure 2. Historic US fuel ethanol production.


○ Ethanol production facility
● Began production in 2002
● Under construction

Source: Renewable Fuels Association

Figure 3. Fuel ethanol plants in the US (source: RFA).

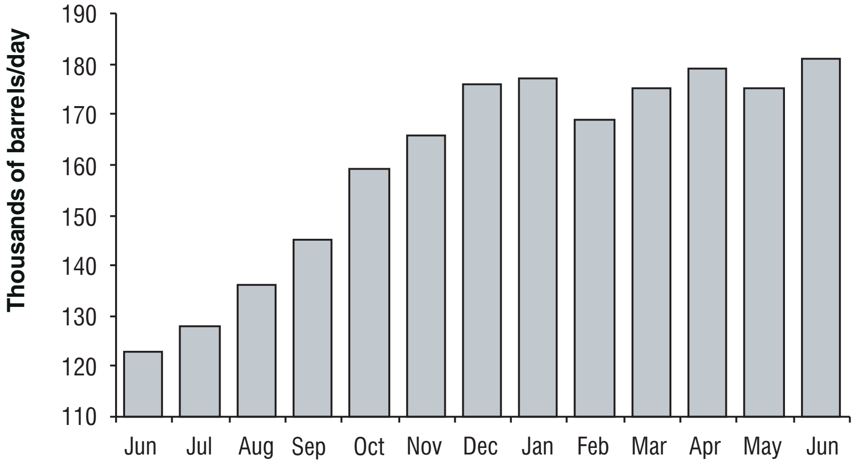


Figure 4. Monthly US ethanol production, 2002-2003 (1 barrel = 42 US gallons or 159 liters).

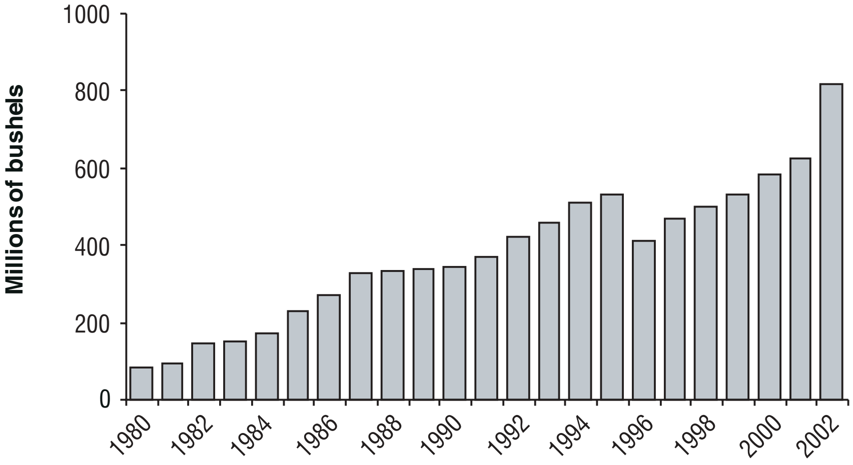


Figure 5. Corn utilized in US ethanol production.

processed into ethanol in 2003, or about 10% of the total US harvest. Since 1999, farmer-owned ethanol plants have increased their percentage of total production capacity from 20% to nearly 35%. As a whole, farmer-owned ethanol plants are the largest ethanol producer in the US.

New marketplace opportunities

Following concerns regarding water contamination with MTBE, the state of California passed landmark legislation phasing out the use of MTBE in the state's motor fuel supply. While the original deadline was delayed by one year to December 31, 2003, all major refiners voluntarily switched to ethanol in early 2003, resulting in a new market for more than 600 million gallons. In 2004, with the MTBE ban in place, California's ethanol consumption will increase to 750-800 million gallons annually.

MTBE bans in New York and Connecticut are also scheduled to take effect on January 1, 2004. These two states combined represent a market for more than 500 million gallons of ethanol. If all of the northeast were to replace current MTBE use with ethanol, a market for more than a billion gallons of ethanol would be created. In total, 17 states have passed legislation to phase out MTBE use; and the future for ethanol looks bright.

Comprehensive energy legislation offers opportunities for ethanol

The nation's increasing reliance on imported energy, particularly from unstable regions of the world, combined with erratic natural gas and world oil markets has prompted the administration and US Congress to debate comprehensive energy policy legislation. As part of this legislation, an historic fuels agreement supported by an unprecedented coalition of agriculture, oil and environmental interests would provide a growing market for renewable fuels while addressing concerns regarding MTBE water contamination and providing needed flexibility in the fuels marketplace. This agreement would establish a Renewable Fuel Standard (RFS), which would set an annual and increasing minimum level of renewable fuels such as ethanol and biodiesel permitted in the marketplace, growing to 5 billion gallons by 2012.

New uses for ethanol

While ethanol today is largely used as a blending component with gasoline to add octane and oxygen, opportunities abound for ethanol to play a role in advanced vehicle technology and alternative fuels markets. For example, as a renewable fuel with an established fueling infrastructure in the US, ethanol is an attractive fuel option for the fuel cell market in both power generation and transportation. Research is also underway on E diesel^{TM}, a mixture of ethanol, diesel and a blending agent. And there is a largely untapped market for E85, a blend of 85% ethanol and 15% gasoline, to power the growing number of available flexible fuel vehicles.

Valuable feed co-product production increasing

Record ethanol production means record production of valuable feed co-products for livestock. In 2002, US dry mill ethanol facilities, which account for approximately 65% of ethanol production, produced about four million short tons of distillers dried grains with solubles (DDGS). Ethanol wet mills produced approximately 450,000 US tons of corn gluten meal, 2.5 million tons of corn gluten feed and germ meal, and 530 million pounds of corn oil. With estimates for 2003 production nearly 30% higher than 2002, we can anticipate a parallel increase in production of feed co-products. A waiting market exists in the world's animal feed industry (>600 million tonnes) where traditionally used sources of protein such as animal by-products and fish meal have been either eliminated due to concern surrounding Mad Cow Disease (BSE) or reduced availability and higher prices. The combined protein and energy value of ethanol by-products gives them tremendous potential in animal feeds across the world.

BRAZIL AND SOUTH AMERICA

The US, however, is not the only country experiencing an increase in alcohol production. In Brazil, new initiatives are underway and the inflexible Proalcohol program has been changed. Flex-fuel engines, which run equally well on either pure hydrous alcohol or gasoline, have been launched. This technology can cope with anything from 100% hydrous ethanol to a gasohol mix of 80% gasoline and 20% anhydrous ethanol, adjusting to any change within a fraction of a second. This compares very favorably to the engines in the US, which would normally handle up to 85% anhydrous in a blend and cannot use pure hydrous ethanol. Furthermore, since ethanol sells in Brazil for less than gasoline at the pumps (50-60% of the gasoline price) even taking into account that 30% more ethanol is required to travel the same distance, the new cars are bound to be a success and will drive up ethanol demand.

It is expected that a million cars with Flex-fuel engines could be sold each year. With an average 240 liters (63 gallons) of ethanol per month consumption, this could mean an additional 3 billion liters would be needed over Brazil's current 12-13 billion liters of production.

Table 1. Projected production of ethanol (billion liters)[1].

	2003	*2005*
USA	10	18
Brazil	13	16
European Union	Unknown	8
India	1.5	1.8
Thailand	Unknown	0.7
Australia	.04	.4
China	Unknown	2

[1] F.O. Licht, July 2003

While few people will say exactly how much it costs to produce a liter of fuel ethanol in Brazil, it is believed to be between $0.10 and $0.15. This puts the industry in a very favorable position to export ethanol, since a similar cost in the US from corn would be more like $0.20.

Fuel ethanol emergence in South America is not limited to Brazil. In Peru and Colombia interest is also growing. Colombia estimates an annual need for almost one billion liters and 12 production plants.

THE EUROPEAN UNION

Driven by the Kyoto Agreement, the European Commission proposed in 2001 to increase the use of crop-based, non-polluting renewable fuels. The target was for biofuels to account for 2% of all fuels in EU transport by 2005. It was mandated this would increase to 5.75% by 2010 (from 0.3% in 2001).

Both ethanol and biodiesel (from canola) are considered biofuels. In 2003 the targets were made voluntary, but a direction on tax relief for renewable energy was expected to be put into place. These would augment the existing tax relief schemes, which vary from country to country. Spain, Germany, and Sweden, for example, have 100% tax relief on renewable fuels, while France has 60% and Britain 40%.

Across Europe, from Poland to Ireland, interest is rising as the new legislation begins to take effect.

ASIA

India's transport sector accounts for over half of the country's oil consumption. It is not surprising, therefore, that interest is growing in replacing dependence on fossil fuels. By January 2003, nine states had mandated at least 5% ethanol (E5) in transport fleets. Local distilleries with a capacity of some 3.2 billion liters (900 million gallons) are currently only operating at 50% capacity. The demand for an additional 400 million liters under this new mandate is therefore immediately being met. Although some political maneuvering is still needed, the fuel ethanol program looks sound.

In Southeast Asia, Thailand's government, like many in the region, had an early interest in bioethanol as a way to reduce oil imports. It was estimated that 0.7 billion liters of ethanol would be required should they decide to introduce the oxygenate at 10% of gasoline. Both cassava (tapioca) and molasses were suggested as raw materials and eight projects were approved. At this time only one project is under construction.

Two distinct areas for ethanol production exist in China: the maize-rich north and the sugarcane rich south. Both will feature in the country's ethanol future. The government sees ethanol as part of its program to reduce pollution – important with the looming 2004 Olympics – and at the same time encourage rural employment. China's largest fuel ethanol facility, costing almost $300 million to construct, is in Jilin. With a capacity of some 600 million liters (165 million gallons), it will use 2 million tons of maize a year (13% of production in that province). By October 2003, all vehicles in the Province of Jilin will use fuel containing at least 10% ethanol.

AUSTRALIA

Uncertainty also exists in Australia, where a target of 350 million liters was encouraged with tax exemptions and grants for up to six years. Local governments have tried to boost ethanol consumption. For example, in Cairns, Queensland's largest city, government-owned vehicles were mandated to use E10 (90% gasoline, 10% ethanol). Using some 20,000 liters per day, the government hoped in this way to avoid some of the negative publicity being generated by the anti-ethanol lobby.

Conclusions

Ethanol, as a global product, is experiencing a significant jump in production and production capacity. Whether it be used domestically or exported to areas around the world (such as Japan, Singapore and even California) where production is not sufficient or not possible, it is clear it will become a global commodity. The future, therefore, is very positive; and this future will not be one dependent on subsidies. A 1997 study by the Kellogg School of Management at Northwestern University concluded the net effect of ethanol production was a reduction in the US federal budget deficit. This was brought about by increased tax revenues due to the increases in personal income from wages and salaries, higher farm income, more corporate tax revenue, and declining unemployment compensation payments. Alcohol as a fuel is clearly with us to stay.

Raw material handling and processing

Chapter 2

Grain dry milling and cooking procedures: extracting sugars in preparation for fermentation

DAVE R. KELSALL AND T. PEARSE LYONS

Alltech Inc., Nicholasville, Kentucky, USA

This chapter deals with the milling and cooking stages of alcohol production from whole cereals. In brief, in this dry milling process the whole cereal, normally corn (maize), is ground in a mill to a fine particle size and mixed with liquid, usually a mixture of water and backset stillage. This slurry is then treated with a liquefying enzyme to hydrolyze the cereal to dextrins, which are a mix of oligosaccharides. The hydrolysis of starch with the liquefying enzyme, called α-amylase, is done above the temperature of gelatinization of the cereal by cooking the mash at an appropriate temperature to break down the granular structure of the starch and cause it to gelatinize. Finally the dextrins produced in the cooking process are further hydrolyzed to glucose in a saccharification process using the exoenzyme glucoamylase, and possibly another enzyme (Rhizozyme™). These enzymes may also be added to the yeast propagation tank or the fermentor. These separate stages of milling, cooking and saccharification will be explained in more detail. This is called the dry milling process. Most of the new distilleries use this process or a minor variation of it.

Milling

The purpose of milling is to break up the cereal grains to appropriate particle size in order to facilitate subsequent penetration of water in the cooking process. The optimum size of the ground particle is the subject of disagreement. Some engineers believe the particle should be as fine as possible to allow the water maximum access for hydrolysis of starch while others believe a better yield is obtained if the particles are larger and the jet cooker can act on the particles. The key is simply to expose the starch without grinding so fine as to cause problems in co-product recovery. A wide variety of milling equipment is available to grind the whole cereal to a meal. Normally, most distilleries use hammer mills, although some may use roller mills, particularly for small cereal grains.

HAMMER MILLS

In a hammer mill, the cereal grain is fed into a grinding chamber in which a number of hammers rotate at high speed. The mill outlet contains a retention screen that holds back larger particles until they are broken down further so that there will be a known maximum particle size in the meal. The screens are normally in the size range of 1/8-3/16 in. (2-4 mm).

Sieve analysis (particle size distribution) shows whether the hammer mill screens are in good order and whether the mill is correctly adjusted, and should be conducted regularly. Table 1 shows a typical sieve analysis for corn. The two largest screens retain only 11% of the particles while the quantity passing through the 60-mesh screen is also fairly low at 7%. For efficient processing of the cereal starch into alcohol, the particles should be as fine as possible. However a compromise must be made such that the particles are not so fine that they cause balling in the slurry tank or problems in the by-product recovery process. Sometimes the sieve analysis will vary depending on the severity of the shaker and a consistent shaking should be done each time the test is done.

Table 1. Typical results of a sieve analysis of corn meal.

Screen size	*Hole size (in)*	*Corn on screen (%)*
12	0.0661	3.0
16	0.0469	8.0
20	0.0331	36.0
30	0.0234	20.0
40	0.0165	14.0
60	0.0098	12.0
Through 60		7.0

Fineness of the grind can be significant factor in the final alcohol yield. It is possible to obtain a 5-10% difference in yield between a fine and a coarse meal. Table 2 shows the typical alcohol yield from various cereals. It can be seen that the normal yield from corn is 2.85 gallons of anhydrous ethanol per bushel (56 lbs). However, the yield with coarsely ground corn may drop to 2.65 gallons per bushel, a reduction in yield of 7.5%. This is a highly significant reduction and would have serious economic consequences for any distiller. Grinding is about expanding the amount of surface area. When more surface area is exposed, water and enzymes are better able to penetrate the kernel. A particle 1 in. in diameter has a total surface area of 6 in^2. If you grind the 1 in. particle into 1,000 particles, surface area becomes 6,000 in^2. No amount of heating or 'exploding' will overcome poor grind as measured by yield. The key is to expose more surface area, but to do so in a controlled manner.

Since grind is so important, it is recommended that a sieve analysis of the meal be done at least once per shift. The distiller should set specifications for the percentage of particles on each sieve; and when the measured quantity falls outside these specifications the mill should be adjusted. Normally the hammers in a hammer mill are turned every 15 days, depending on usage; and every 60 days a decision should be made as to whether or not to replace the hammers and screens.

Table 2. Typical alcohol yields from various cereals.

Cereal	*Yield*[a] *(US gallons of anhydrous alcohol/bushel)*
Fine grind corn[1], 3/16 in.	2.85
Coarse grind corn[1], 5/16 in.	2.65
Milo	2.60
Barley	2.50
Rye	2.40

[a] Note that a distiller's bushel is always a measure of weight. It is always 56 lb, regardless of the type of grain.

[1] Laboratory data using Rhizozyme™ as glucoamylase source.

Since sieve analysis is critical, a case can also be made for recycling to the hammer mill any grain not ground sufficiently fine. Fineness of the grind also has an important bearing on centrifugation of the stillage post-distillation. A finer grind may yield more solubles and hence place a greater load on the evaporator. However since the key is to maximize yield, dry house considerations, while important, cannot override yield considerations.

ROLLER MILLS

Some distillers use roller mills (e.g. malt whisky producers), particularly where cereals containing substantial quantities of husk material are used. In a roller mill, the cereal is nipped as it passes through the rollers, thus exerting a compressive force. In certain cases, the rollers operate at different speeds so that a shearing force can be applied. The roller surfaces are usually grooved to aid in shearing and disintegration. Figure 1 shows the general configuration of a roller mill.

In Scotland the solids in whisky mash, made entirely from malted barley, are usually removed by using a brewery-type lauter tun, which is a vessel with a perforated bottom like a large colander. In this case, a roller mill should be used as the shear force allows the husk to be

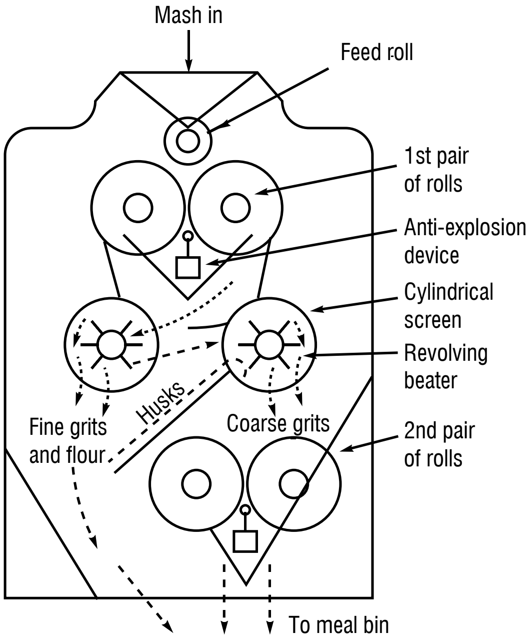


Figure 1. Schematic of a roller mill.

separated with minimal damage. The husk then acts as the filter bed in the lauter tun for the efficient separation of solids and liquid.

Cooking

The purpose of cooking is 5-fold:

Sterilization. The mash must be sterilized so that harmful bacteria are minimized. A brewer achieves this by boiling mash (wort) while the distiller achieves it by cooking.

Solubilization of sugars. Sugars are solubilized to a certain point, typically 2-3% free sugars. In this way yeast growth can occur rapidly but not excessively as would happen if too much sugar was released at once.

Release of all bound sugars and dextrins (chains of sugar). Extraction must occur such that subsequent enzymatic hydrolysis can ensure that all sugars are utilized. Starch (precursor of sugars) is in large bound to protein and fiber, which is are freed during cooking.

Protein breakdown to amino acids should be minimized. Amino acids and peptides can become bound to sugars in Maillard reactions, which leaves sugar unavailable.

Reduction in viscosity. Following gelatinization, viscosity is reduced allowing the slurry to be moved through lines for subsequent processing.

Cooking is the entire process beginning with mixing the grain meal with water (and possibly backset stillage) through to delivery of a mash ready for fermentation. Figure 2 shows the components that make up a typical milling and cooking system. This schematic diagram could represent the processes involved in beverage, industrial or fuel alcohol production, except that nowadays only the whisky distillers use malt as a source of liquefying and saccharifying enzymes. All other alcohol producers use microbial enzyme preparations. The fastest enzyme systems have low calcium requirements (2-5 ppm), lower pH optima (less buffering required) and high temperature stability. They are designed to reduce viscosity, with less emphasis on DE. The key to cooking is to liquefy the starch so it can be pumped to the fermentor, hence viscosity is critical.

HYDROLYSIS OF STARCH TO FERMENTABLE SUGARS

Step 1: gelatinization

The purpose of cooking and saccharification is to achieve hydrolysis of starch into fermentable sugars, which is accomplished by the endoenzyme α–amylase, followed by the exoenzyme glucoamylase (amyloglucosidase) to release glucose. However in order for the α-amylase to gain access to the starch molecules, the granular structure of the starch must first be broken down in the process known as *gelatinization*. When the slurry of meal and water are cooked, the starch granules start to adsorb water and swell. They gradually lose their crystalline structure until they become large, gel-filled sacs that tend to fill all of the available space and break with agitation and abrasion.

The peak of gelatinization is also the point of maximum viscosity of a mash. Figures 3, 4 and 5 show the progressive gelatinization of cornstarch, as viewed on a microscopic hot stage. In Figure 3 the granules are quite distinct and separate from the surrounding liquid. In Figure

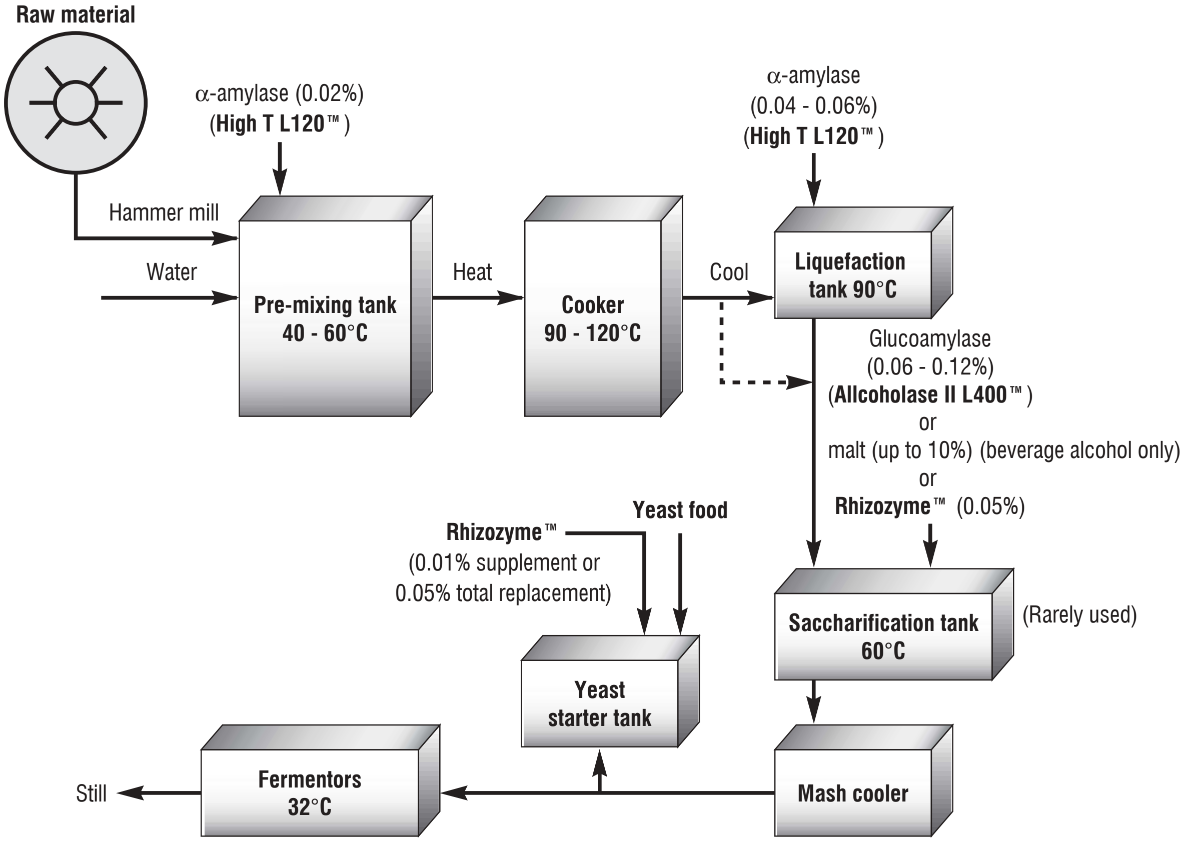

Raw material
α-amylase (0.02%)
(High T L120™)
α-amylase
(0.04 - 0.06%)
(High T L120™)
Hammer mill
Water
Pre-mixing tank
40 - 60°C
Heat
Cooker
90 - 120°C
Cool
Liquefaction
tank 90°C
Glucoamylase
(0.06 - 0.12%)
(Allcoholase II L400™)
or
malt (up to 10%) (beverage alcohol only)
or
Rhizozyme™ (0.05%)
Yeast food
Rhizozyme™
(0.01% supplement or
0.05% total replacement)
Saccharification tank
60°C
(Rarely used)
Yeast
starter tank
Still
Fermentors
32°C
Mash cooler

Figure 2. Components of a milling and cooking system.

4 these same granules have swollen in size and some of the liquid has entered the granules. Figure 5 shows the granules as indistinct entities in which the liquid has entered to expand them considerably.

Gelatinization temperatures vary for the different cereals (Table 3). Some distillers consider it important for the slurrying temperature of the meal to be below the temperature of gelatinization. This avoids coating of grain particles with an impervious layer of gelatinized starch that prevents the enzymes from penetrating to the starch granules and leads to incomplete conversion. Many distillers, however, go to the other extreme and slurry at temperatures as high as 90°C (190°F). At these temperatures starch gelatinizes almost immediately and with adequate agitation there is no increase in viscosity and no loss of yield.

Table 3. Gelatinization temperature ranges of various feedstocks.

	Gelatinization range (°C)
Corn	
Standard	62-72
High amylose 1	67->80
High amylose 2	67->80
Barley	52-59
Rye	57-70
Rice (polished)	68-77
Sorghum (milo)	68-77
Wheat	58-64

Step 2. Liquefaction: hydrolysis of starch to dextrins

Liquefaction is accomplished by the action of α–amylase enzyme on the exposed starch molecules. Starch exists in two forms. One form

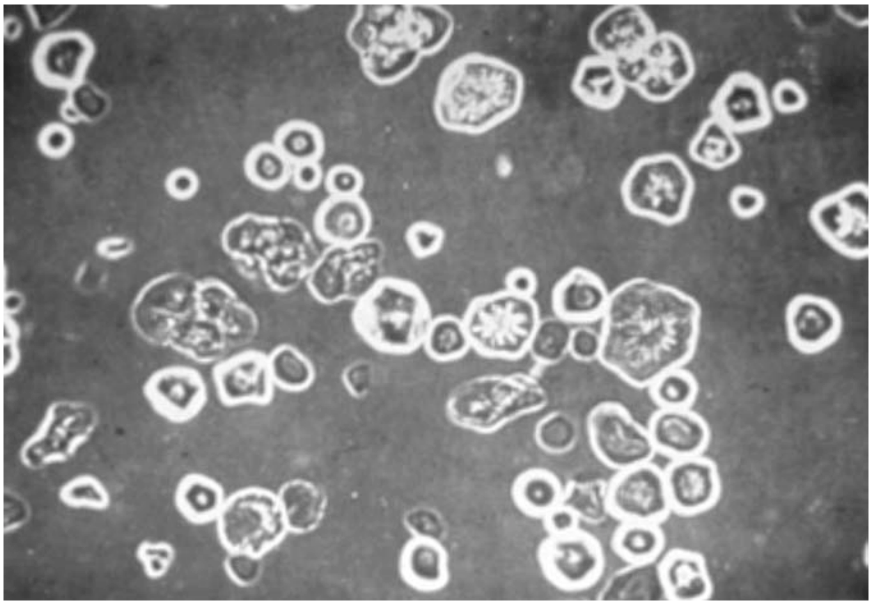


Figure 3. Gelatinization of cornstarch. Starch granules viewed on microscope hot stage at 67°C (under normal illumination).

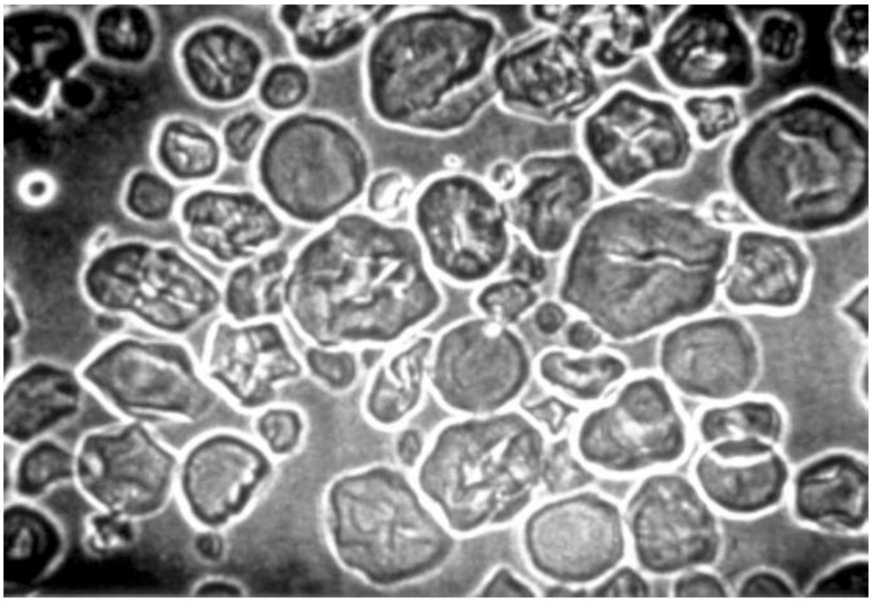


Figure 4. Gelatinization of cornstarch. Same granules as in Figure 3 at 75°C (under normal illumination).

is the straight-chained amylose, where the glucose units are linked by α–1,4 glucosidic linkages (Figure 6). The amylose content of corn is about 10% of the total starch; and the amylose chain length can be up to 1,000 glucose units. The other form of starch is called amylopectin, which represents about 90% of the starch in corn. Amylopectin has a branched structure (Figure 7). It has the same α–1,4 glucosidic linkages as in amylose, but also has branches connected by α–1,6 linkages. The number of glucose units in amylopectin can be as high as 10,000. Corn, wheat and sorghum (milo), the three most common feedstocks for ethanol production, have

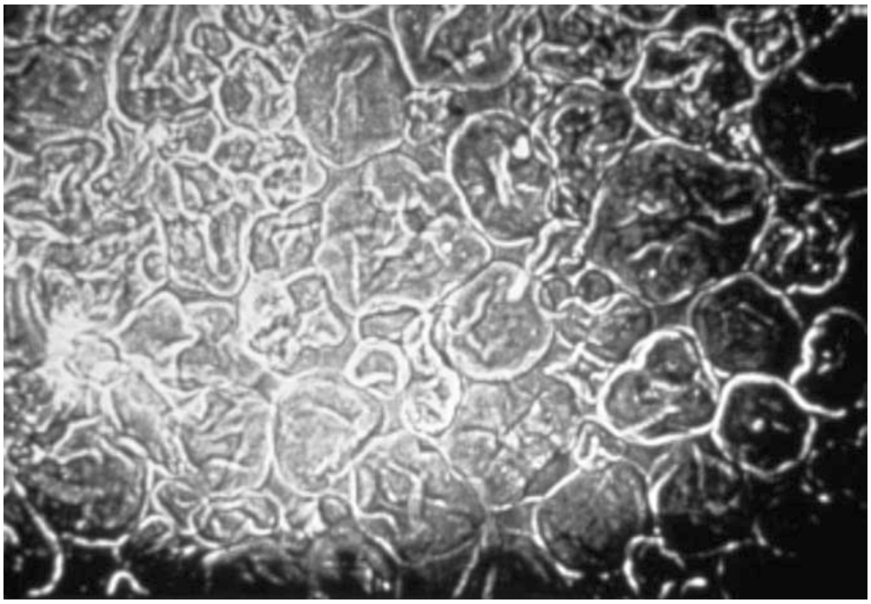


Figure 5. Gelatinization of cornstarch. Same granules as in Figures 3 and 4 at 85°C (under normal illumination).

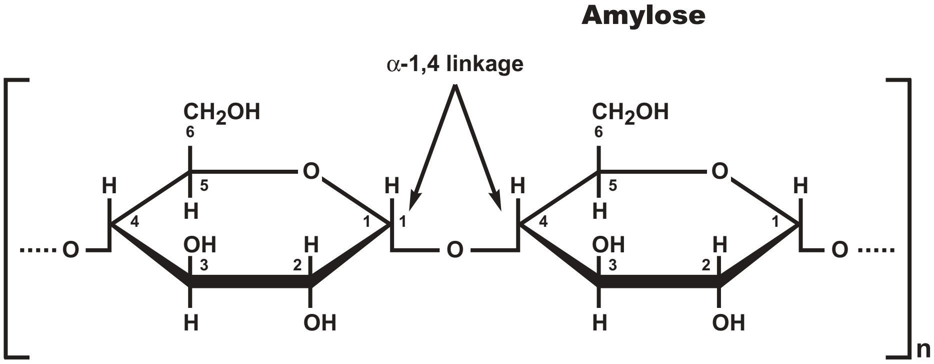


Figure 6. Structure of amylose.

similar levels of starch, but percentages of amylose and amylopectin differ with grain and with variety. Starch structures are reviewed in greater detail in the chapter by R. Power in this volume.

The α–amylase enzyme acts randomly on the α–1,4 glucosidic linkages in amylose and amylopectin but will not break the α–1,6 linkages of amylopectin. The resulting shorter straight chains (oligosaccharides) are called *dextrins*, while the shorter branched chains are called *α-limit dextrins.* The mixture of dextrins is much less viscous.

Step 3. Saccharification: release of glucose from dextrins

Saccharification is the release of the individual glucose molecules from the liquefied mixture of dextrins. The dextrins will be of varying chain lengths. However, the shorter the chain length the less work remaining for the exoenzyme glucoamylase, which releases single glucose molecules by hydrolyzing successive α–1,4 linkages beginning at the non-reducing end of the dextrin chain. Glucoamylase also hydrolyzes α–1,6 branch linkages, but at a much slower

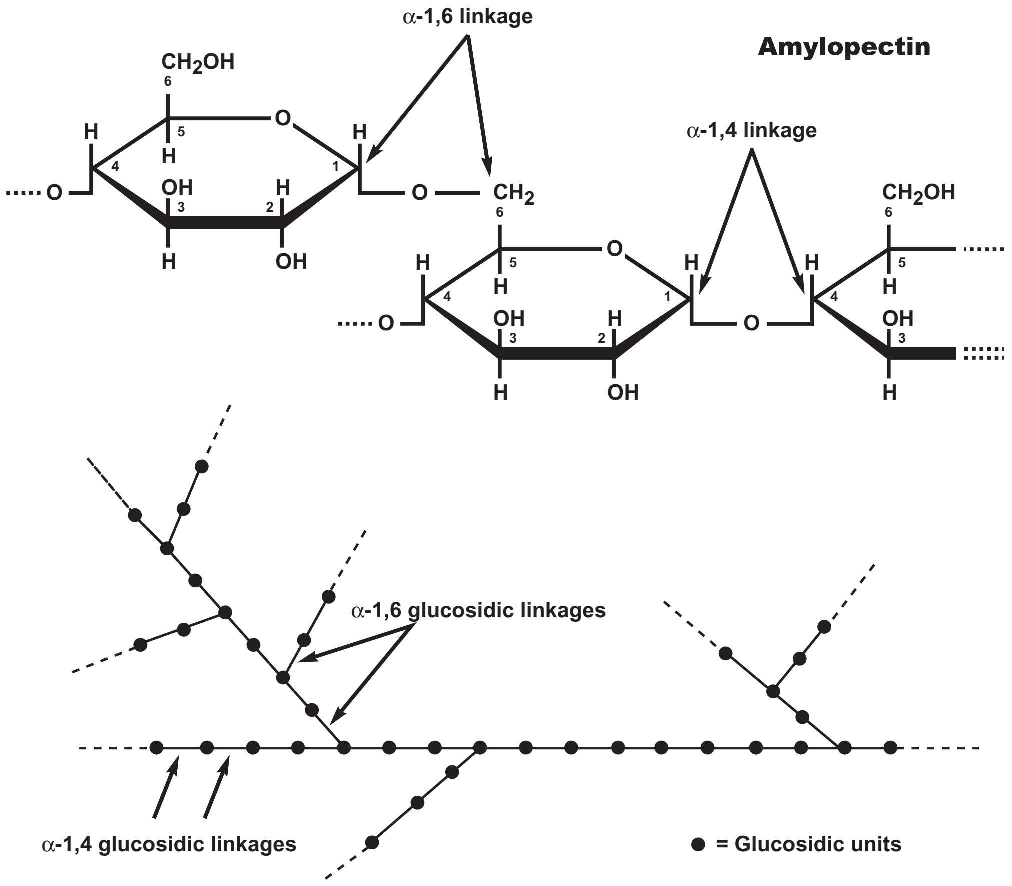


Figure 7. Structure of amylopectin.

rate. One real problem with HPLC analysis is that dextrins are given as total oligosaccharides with no differentiation between four glucose units and 20 glucose units. The work of the glucoamylase is directly proportional to the length of the dextrin chain; and there is currently no way of knowing how well the α-amylase has worked to produce small oligosaccharides.

In Scotch whisky production, malted barley is used as a source of both α–amylase and the exoenzyme ß-amylase. The fermentable sugar produced is maltose, a dimer made up of two glucose units.

Cooking systems

BATCH COOKING SYSTEMS

In considering all the different processes that make up cooking, it should first be explained that there are a variety of batch and continuous cooking systems. For a batch system there is usually only one tank, which serves as slurrying, cooking and liquefaction vessel. Live steam jets are typically installed in the vessel to bring the mash to boiling temperature along with cooling coils to cool the mash for liquefaction. Figure 8 shows a typical batch cooking system.

In the batch cooking system, a weighed quantity of meal is mixed into the vessel with a known quantity of water and backset stillage. These constituents of the mash are mixed in simultaneously to ensure thorough mixing. The quantity of liquid mixed with the meal will determine the eventual alcohol content of the fermented mash. When a distiller refers to a '25 gallon beer', it means 25 gallons of liquid per bushel of cereal. For example, for a corn distillery with an alcohol yield of 2.5 gallons of absolute alcohol per bushel, the 25 gallons of

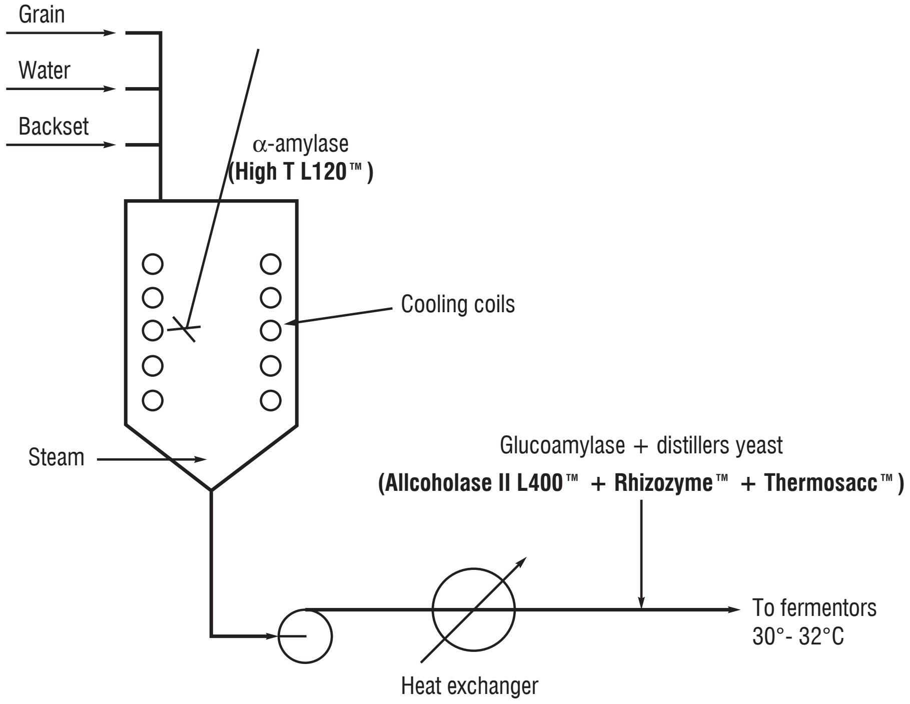


Figure 8. Batch cooking system.

liquid would contain 2.5 gallons of alcohol. Therefore it would contain 10% alcohol by volume (abv). Using the distillery alcohol yield, the distiller can determine the quantity of cereals and liquid to use. Most distilleries traditionally operated with beers in the 10-15% abv range, although some beverage plants run at alcohol levels as low as 8%. More recently fuel alcohol plants run at alcohol levels as high as 19-20% by volume, the average being nearer to16-17% by volume.

In the batch cooking system, a small quantity of α–amylase is added at the beginning (0.02% w/w of cereal) to facilitate agitation in the high viscosity stage at gelatinization. After boiling, usually for 30-60 minutes, (sometimes under a slight pressure), the mash is cooled to 75-90°C and the second addition of α–amylase made (0.04-0.06% w/w cereal). Liquefaction then takes place, usually over a holding period of 45-90 minutes. The mash should always be checked at this stage to make certain that no starch remains. Starch produces a blue or purple color with iodine. Mash should not be transferred from the liquefaction hold until it is 'starch-negative'.

The pH range for efficient α–amylase usage is 6.0-6.5, although enzymes with good activity at pH 5.5-5.7 are now available. Therefore, mash pH should be controlled in this range from the first enzyme addition until the end of liquefaction. The glucoamylase enzyme has a lower pH range (4.0-5.5), so after liquefaction the pH of the mash should be adjusted with either sulfuric acid or backset stillage, or a combination of the two.

The quantity of backset stillage as a percentage of the total liquid varies from 10 to 50%. On one hand, the backset stillage supplies nutrients essential for yeast growth. However too much backset stillage can result in the oversupply of certain minerals and ions that suppress good fermentation. Especially noteworthy are the sodium, lactate and acetate ions. Sodium concentrations above 0.03% or lactate above 0.8% or acetate above 0.03% can inhibit yeast growth and can slow or possibly stop the fermentation prematurely. Overuse of backset (or even process condensate water) must be avoided to prevent serious fermentation problems.

CONTINUOUS COOKING SYSTEMS

Few distilleries outside of beverage plants use batch cooking. Most fuel ethanol distilleries use a continuous cooking system. In the continuous cooking process (Figure 9) meal, water and backset stillage are continuously fed into a premix tank. The mash is pumped continuously through a jet cooker, where the temperature is instantly raised to 120°C. It then passes into the top of a vertical column. With plug flow, the mash moves down the column in about 20 minutes and passes into the flash chamber for liquefaction at 80-90°C. High temperature-tolerant α–amylase is added at 0.04-0.08% w/w cereal to bring about liquefaction. The retention time in the liquefaction/flash chamber is a minimum of 30 minutes, but should be at least 60 minutes. The pH from slurrying through to the liquefaction vessel must be controlled within the 6.0-6.5 range. The greatest advantage of this system is that no enzyme is needed in the slurrying stage, leading to significant savings in enzyme usage. It is critical to have plug flow through the chamber along with good enzyme dispersion. The mash should have a relatively low viscosity and dextrose level of 2-3%. Modern systems have 29-33% solids. From the liquefaction chamber, the mash is pumped through a heat exchanger to fermentation.

CONTINUOUS U-TUBE COOKING SYSTEM

The continuous U-tube cooking system (Figure 10) differs from the columnar cooking system in that the jet cooker heats the mash to 120-140°C prior to being transferred through a continuous U-tube. The retention time in the U-tube is only three minutes, after which it is flashed into the liquefaction vessel at 80-90°C and the enzyme is added (high temperature-tolerant α-amylase 0.05-0.08% w/w cereal). The residence time in the liquefaction vessel is a minimum of 30 minutes.

The main advantage of this system is the relatively short residence period in the U-tube. If properly designed there is no need to add any α–amylase enzyme in the slurrying stage. However, because of the relatively narrow diameter of the tubes, some distillers add a small amount of enzyme to the slurry tank to guarantee a free flow.

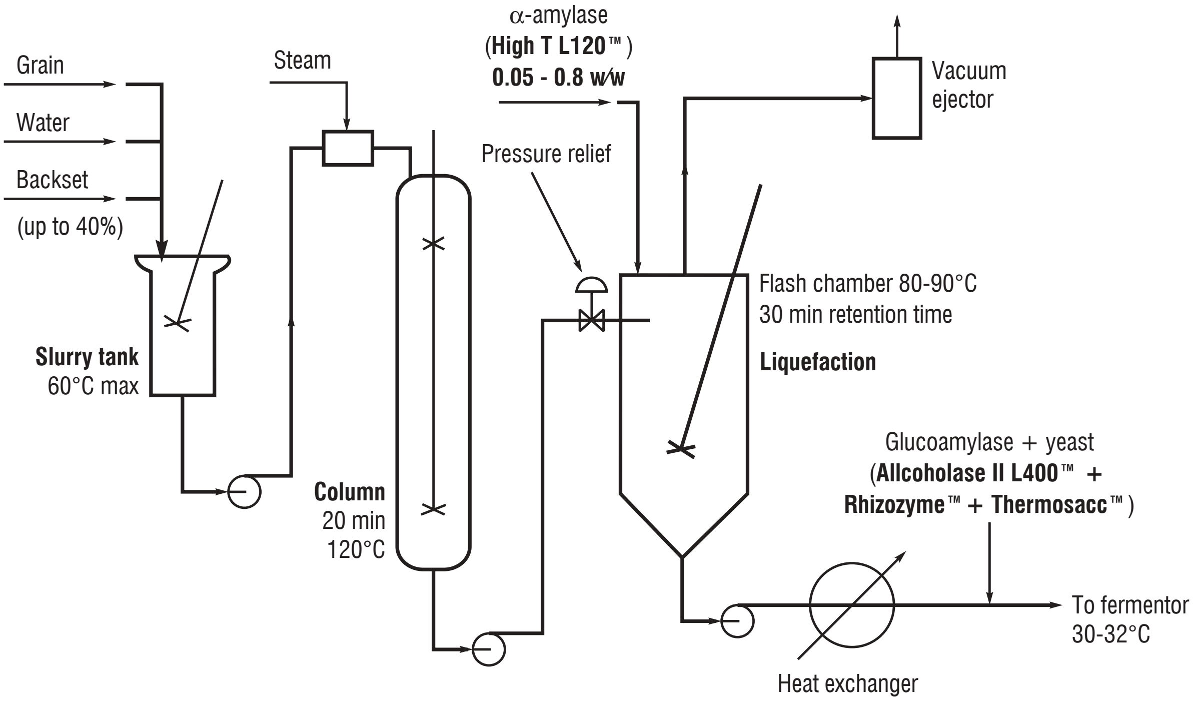

Grain
Water
Backset
(up to 40%)
Slurry tank
60°C max
Steam
Column
20 min
120°C
α-amylase
(High T L120™)
0.05 - 0.8 w/w
Pressure relief
Vacuum
ejector
Flash chamber 80-90°C
30 min retention time
Liquefaction
Glucoamylase + yeast
(Allcoholase II L400™ +
Rhizozyme™ + Thermosacc™)
To fermentor
30-32°C
Heat exchanger

Figure 9. Continuous columnar cooking system.

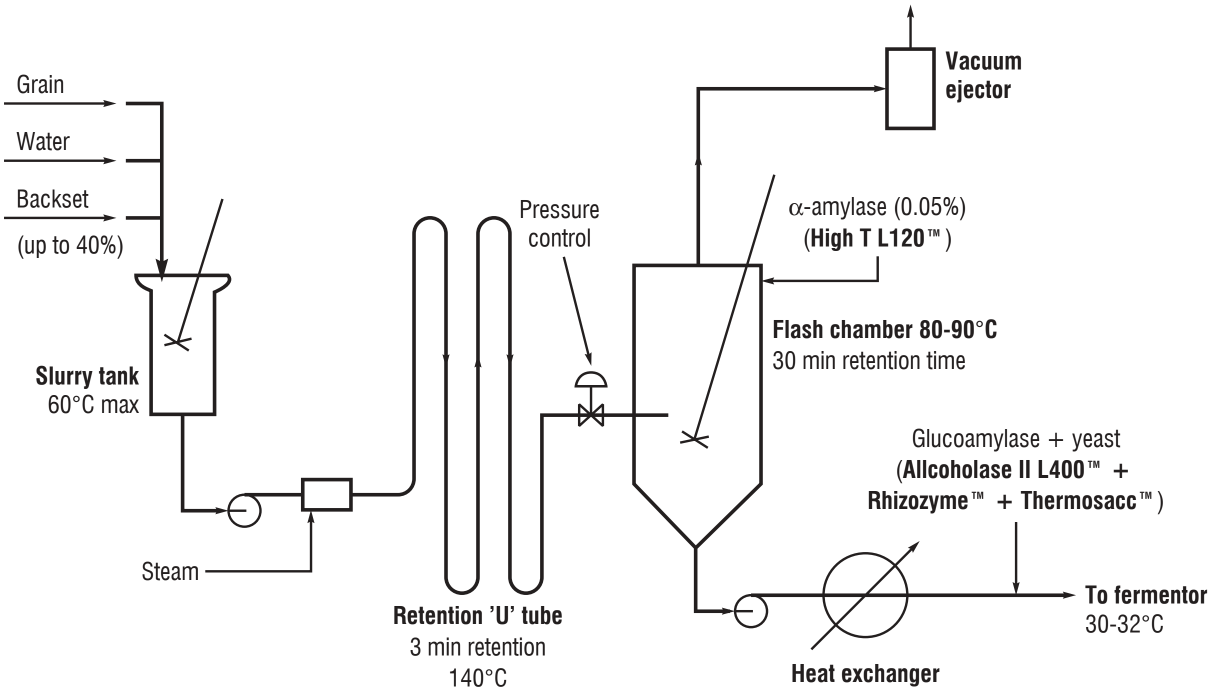

Grain
Water
Backset
(up to 40%)
Slurry tank
60°C max
Steam
Pressure control
Retention 'U' tube
3 min retention
140°C
Vacuum ejector
α-amylase (0.05%)
(High T L120™)
Flash chamber 80-90°C
30 min retention time
Glucoamylase + yeast
(Alcoholase II L400™ + Rhizozyme™ + Thermosacc™)
To fermentor
30-32°C
Heat exchanger

Figure 10. High temperature, short time, continuous U-tube cooking system.

COMPARING COOKING SYSTEMS

The relative heat requirements of the three cooking systems can be seen in Table 4. Surprisingly, the batch system is the most energy-efficient. Batch systems also generally use less enzyme than the other systems, possibly due to the difficulty of accurate dosing and good mixing with the continuous systems. The main disadvantage of the batch system compared to the continuous system is the poor utilization or productivity per unit of time. The temperature-time sequences for the three systems shown in Figure 11 demonstrate how much more efficiently time is used in continuous systems compared to the batch system.

Table 4. Relative heat requirements of cooking systems.

Batch	1
Continuous columnar	1.18
Continuous U-tube	1.37

In the continuous systems, the flow diagrams show steam addition to raise the mash temperature. This temperature increase is brought about instantaneously by a jet cooker or ‘hydroheater’.

One purpose of the cooking process is to cleave the hydrogen bonds that link the starch molecules, thus breaking the granular structure and converting it to a colloidal suspension. Another factor in the breakdown of starch is the mechanical energy put into the mash via agitation of the different vessels in which the cooking process takes place. Well-designed agitation is very important in a cooking system; and the problem is intensified when plug flow is also desired.

Mash viscosities give an indication of the relative ease or difficulty with which some cereals are liquefied. Figure 12 compares viscosity against temperature for corn and waxy maize (amioca) and illustrates the difference in viscosity profiles.

All of the cooking systems described require the addition of enzymes at least for the liquefaction stage where most of the hydrolysis takes place. Many distilleries now use a high temperature-tolerant α–amylase. The optimum pH range for this enzyme is between 5.8 and 6.5, although it shows good stability up to pH

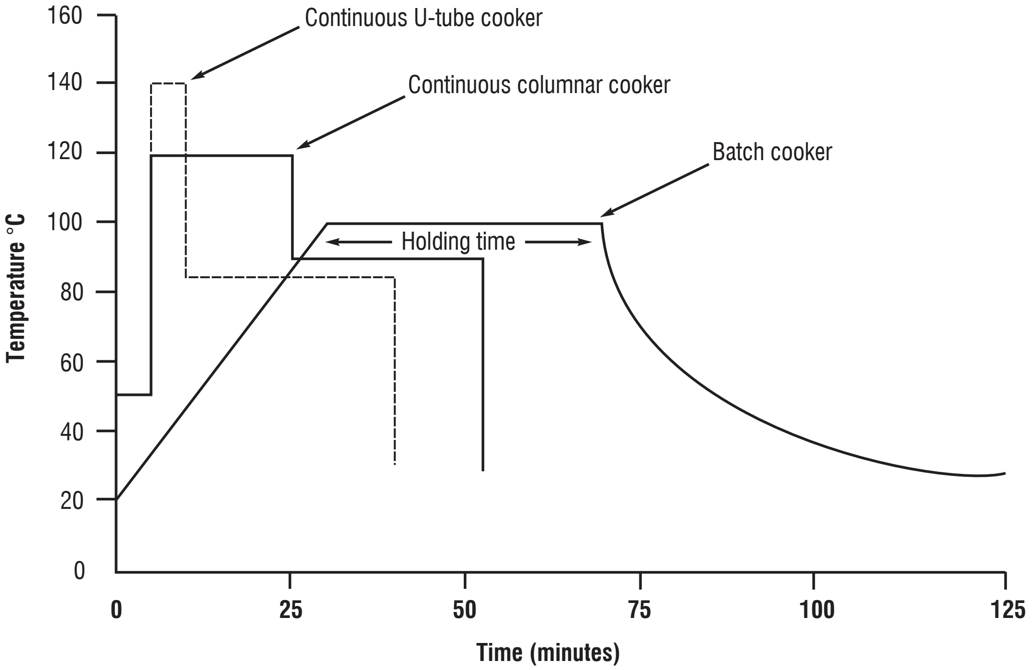


Figure 11. Temperature/time sequences in various types of cooking systems.

8.5 (Figure 13) while the optimum temperature range is 88°C-93°C (Figure 14). Typically, this type of enzyme would be used at between 0.04% and 0.08% by weight of cereal. Where it is necessary to add some α–amylase enzyme to the slurrying vessel, the dosage rate may be slightly higher.

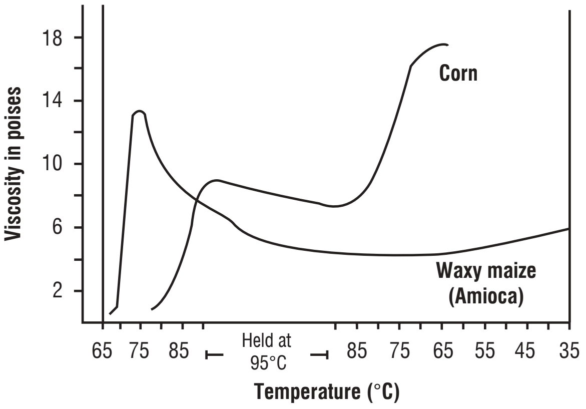


Figure 12. Increasing viscosity with cooking temperature.

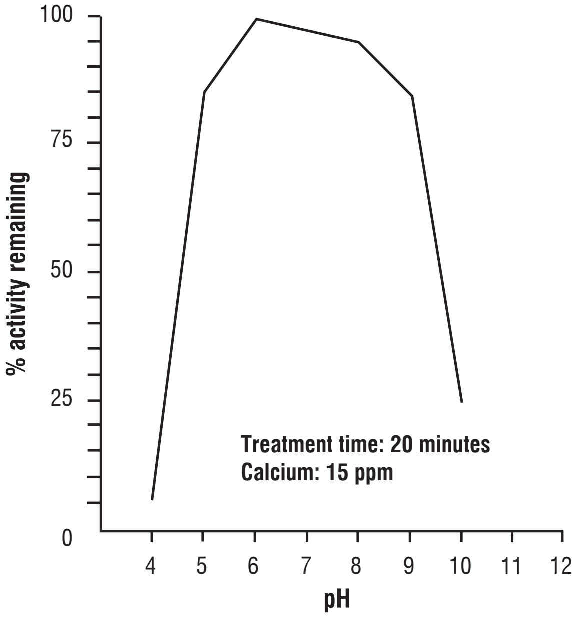


Figure 13. Effect of pH on activity of α-amylase.

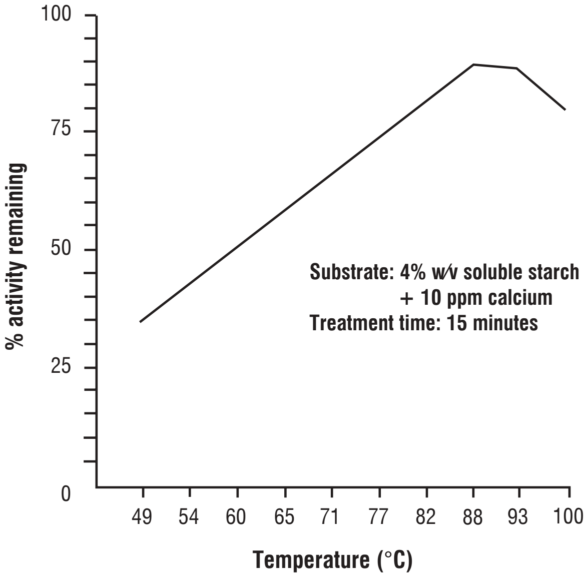


Figure 14. Activity of high temperature-tolerant α-amylase in relation to temperature.

The reaction time for enzyme-catalyzed reactions is directly proportional to the concentration of enzyme. Consequently, distillers wishing to minimize the quantity of enzyme used should design equipment to have long residence times to allow the reactions to be completed with minimal dosage of enzyme.

Saccharification: should we have high levels of sugar going into the fermentor?

Saccharification of distillery mashes is a somewhat controversial subject. Over the last 10 years many distillers have changed from saccharifying mash in a dedicated saccharification vessel (or 'sacc' tank) to adding the saccharifying enzyme directly to the fermentor in a process referred to as Simultaneous Saccharification and Fermentation (SSF). Saccharification in a separate vessel is still the practice in some distilleries, particularly for beverage production and continuous fermentation.

Another factor is use of Rhizozyme™, an enzyme produced by surface culture (solid state fermentation). Rhizozyme™ has an optimum pH of 3.5-5.0 and an optimum temperature of 30-35°C. As such, it is a glucoamylase more suited to a distillery fermentor (Table 5). Rhizozyme™ has particularly found favor in SSF situations because the enzyme can work at near its optimum in the fermentor. Because the organism used to produce Rhizozyme™ is grown on a solid substrate (eg wheat bran), this enzyme source also contains side activities that assist in releasing more carbohydrate and protein (Table 5). These side activities work to release sugars from other structural carbohydrates besides starch, which also releases bound starch that is then available for conversion. This approach to enzyme addition is able to increase yield to higher levels than normally seen with single activity liquid glucoamylase. Levels in excess of 2.9 gallons/bushel have been reported; and the target is 3.1 gallons/bushel (122 gallons or 456 liters per tonne).

Table 5. Advantages of Rhizozyme™ over conventional glucoamylase.

	Conventional glucoamylase	*Rhizozyme™*
Temperature optimum, °C	60+	30+
pH optimum	4.0-5.5	3.5-5.0
Amylase activity, SKB units	None	50,000
Cellulase activity CMC-ase units	None	2500
Amylopectinase, AP units	None	5000

IF A SACCHARIFICATION STEP IS USED

Mash from the liquefaction vessel is cooled, usually to 60-65°C, and transferred to a saccharification vessel where the glucoamylase (amyloglucosidase) enzyme is added. This exoenzyme starts hydrolyzing the dextrins from the non-reducing end of the molecule and progressively, though slowly compared to endoenzymes, releases glucose. The saccharifying process is usually carried out with a residence time of between 45 and 90 minutes, but can be as long as 6 hrs, and the glucoamylase is added at 0.06-0.08% by weight of cereal used. Some distillers measure the quantity of glucose produced by measuring the dextrose equivalent (DE) of the mash. A DE of 100 represents pure glucose, while zero represents the absence of glucose. This test is rarely used nowadays, as many distillers have high performance liquid chromatography systems (HPLC) that can measure sugars directly. Recent experience, however, shows that DE, provided it is above 10, is of no concern. In focusing on 23% ethanol, the key in any cooking and liquefaction process is to liquefy, i.e., lower viscosity, so that mash can be pumped through the heat exchanger to the fermentor (for SSF) or to the saccharification tank.

The functional characteristics of liquid glucoamylase prepared from the microorganism *Aspergillus niger*, can be seen in Figures 15 and 16. Two parameters, temperature and pH, dictate how enzymes can be used. While liquefaction is carried out at a pH of 6.0-6.5 and a temperature of 90°C, this is not at all acceptable for saccharification. The pH must be in the 4-5 range for saccharification; and the optimum temperature for the glucoamylase activity is 60°C. The mash, therefore, must be acidified with either sulfuric acid or backset stillage or both before addition of the glucoamylase. Temperature must also be adjusted. As mentioned previously, normal mash saccharification temperature is 60-65°C; although for microbiological reasons 70-75°C would be preferable. *Lactobacillus* can survive at 60°C; and frequent infection of saccharification systems has caused many distillers to change to saccharifying in the fermentor.

IF NO SACCHARIFICATION STEP IS USED

If no saccharification step is planned, the liquefied mash is simply cooled from 90°C through a heat exchanger and transferred to the fermentor. A portion of the liquefied mash is diverted to a yeast starter tank where yeast, glucoamylase and the Rhizozyme™ is added. Conventional glucoamylase (L400) is added at 0.06% with 0.01% Rhizozyme™ recommended as a supplement. Rhizozyme™ alone can be added at 0.05%, in which case no glucoamylase is required.

Recommendations

Given the goals and the variables involved, which cooking system should be chosen? A comparison of systems used in four distilleries demonstrates the diversity of approach possible, yet points out many similarities (Table 6). It would appear that there are three schools of thought:

a) A long liquefaction period (1-2 hrs at 90+ °C) after the high temperature jet cooker and low temperature slurry (no enzymes).

b) A short high temperature slurry prior to a long liquefaction period (1-2 hrs at 90+ °C) after the high temperature jet cooker. Enzymes used in slurry.

c) Choice of saccharification stage or SSF.

From the experience of the authors, recommendations are for a short slurry at 140-150°F followed by jet cooking (10-15 min.) and a long (1.5 hrs) liquefaction step. The mash should have a sugar profile similar to Table 7. It should then be cooled to fermentation temperature on the way to the fermentor. This profile will ensure a rapid start by yeast with no sugar overload. In the presence of Rhizozyme™ or glucoamylase, dextrins will continue to be 'spoon-fed' to the yeast during fermentation. Enzyme additions should include all the amylase during liquefaction and Rhizozyme™ during fermentation.

Table 7. The target sugar profile following liquefaction.

pH	4.2
Solids	29-35
Glucose, %	1.8-2.5
Maltose, %	0.7-0.9
Maltotriose, %	0.2-0.3
Lactic, %	0.07-0.08
Glycerol, %	0.2-0.3

The future

Many new enzyme systems including xylanases, hemicellulases, ligninases (esterases) and others are being developed and the cooking system must be flexible enough to handle them. The objective is to maximize the biochemical activity of the enzyme so as to maximize alcohol yield, not to fit an enzyme to an existing engineering design.

Chapter 3

Enzymatic conversion of starch to fermentable sugars

RONAN F. POWER

North American Biosciences Center, Alltech Inc., Nicholasville, Kentucky, USA

Introduction

Starch is the second most abundant carbohydrate in the plant world after cellulose. It is the principal plant storage polysaccharide and is similar in structure and function to glycogen, which is the main storage polysaccharide of the animal kingdom. Both starch and cellulose are glucose-based polymers that differ in the orientation of the linkages between the glucose subunits in the respective macromolecules. *Saccharomyces cerevisiae* is the primary microorganism involved in the transformation of starch to ethyl alcohol, and corn starch remains the major raw material for industrial alcohol fermentation although potato starch also has limited use, particularly in Europe. For example, in the 1999 US crop year, 526 million bushels of corn (14.7 million tons at 15% moisture) were used in the fuel ethanol industry (McAloon *et al*., 2000).

The starting compound for alcohol synthesis by yeast is glucose (dextrose), which is a six-carbon sugar. Once fermented, each molecule of glucose yields two molecules of ethanol and carbon dioxide, respectively.

$$C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2$$

The sequence of biosynthetic reactions by which yeast converts glucose to ethanol is termed the Embden-Meyerhof-Parnas (glycolytic) pathway (Figure 1). In this pathway, glucose is phosphorylated, then split into two phosphorylated 3-carbon derivatives, glyceraldehyde-3-phosphate and dihydroxyacetone phosphate. Both products are subsequently converted into pyruvic acid, which under anaerobic conditions yields ethanol and carbon dioxide. It is obvious from Figure 1 that the yeast, *S. cerevisiae,* is very well equipped to ferment glucose and fructose. This is why wine fermentations are technically very simple. Grape pressings deliver pure glucose and, of course, the required yeast are already present on the surface of the grapes. Likewise, molasses represents a readily assimilated feedstock for yeast since the predominant sugar in molasses is the disaccharide, sucrose. Yeast contains the enzyme sucrase, which cleaves sucrose into its constituent monosaccharides, glucose and fructose.

Starch, however, cannot be fermented directly by *S. cerevisiae*, because the organism lacks the requisite starch-degrading or amylolytic enzymes to liberate glucose from this storage polysaccharide. Whether the starch is derived from corn, potatoes, sorghum (milo), barley or other cereal grains, the bonds between the glucose subunits in the starch chain must first

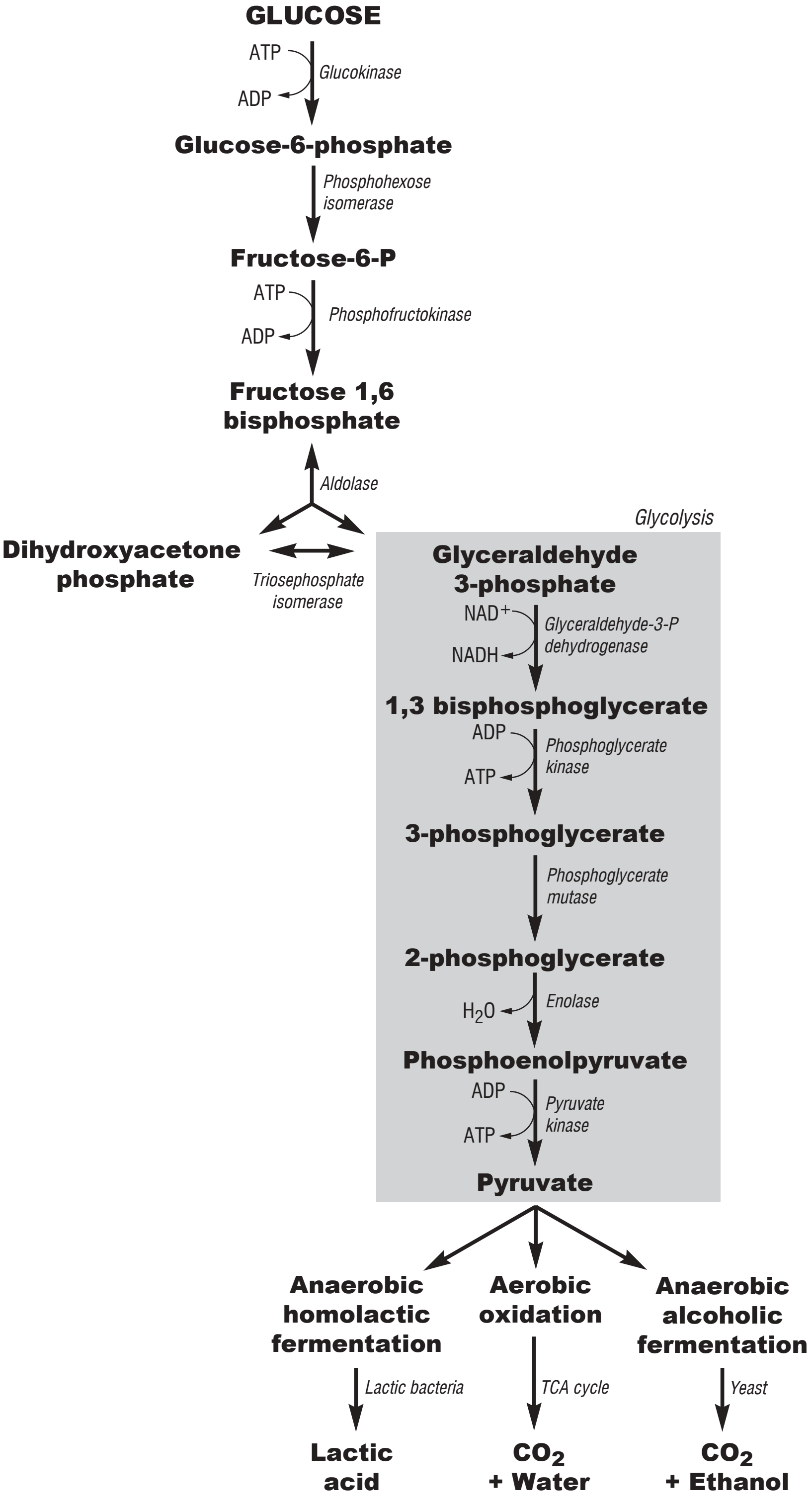

GLUCOSE
ATP
ADP
Glucokinase
Glucose-6-phosphate
Phosphohexose isomerase
Fructose-6-P
ATP
ADP
Phosphofructokinase
Fructose 1,6 bisphosphate
Aldolase
Glycolysis
Dihydroxyacetone phosphate
Triosephosphate isomerase
Glyceraldehyde 3-phosphate
NAD+
NADH
Glyceraldehyde-3-P dehydrogenase
1,3 bisphosphoglycerate
ADP
ATP
Phosphoglycerate kinase
3-phosphoglycerate
Phosphoglycerate mutase
2-phosphoglycerate
H2O
Enolase
Phosphoenolpyruvate
ADP
ATP
Pyruvate kinase
Pyruvate
Anaerobic homolactic fermentation
Aerobic oxidation
Anaerobic alcoholic fermentation
Lactic bacteria
TCA cycle
Yeast
Lactic acid
CO2 + Water
CO2 + Ethanol

Figure 1. The Embden-Meyerhof-Parnas (glycolytic) pathway for ethanol production by yeast.

be hydrolyzed to liberate free glucose molecules which yeast can utilize. Brewers achieve this by exploiting the endogenous starch-digesting enzymes produced by seeds when they germinate. Grains, such as barley, destined for fermentation, are first sprouted to initiate enzyme synthesis within the seeds. This process is termed malting. The malted grains are then steeped in hot water to allow the enzymes to convert starch to fermentable sugar. This mashing phase is then followed by a lautering or mash filtration stage to separate the solids from the glucose-rich liquid 'wort' to which yeast is added.

The first step in the industrial production of alcohol from starch also requires that the starch is *saccharified*; and this is generally recognized to consist of two phases: a) the dextrinization or *liquefaction* phase in which starch is partially degraded to soluble dextrins, and b) the true saccharification or 'conversion' phase in which the dextrins are hydrolyzed to fermentable sugars. The two phases of saccharification may be carried out by either acid or enzymatic hydrolysis. In the past, acid combined with high temperatures and pressures was used to accomplish the same reaction that enzymes perform today under relatively mild conditions. Acid hydrolysis was extremely hazardous, required corrosion-resistant vessels and resulted in a product with a very high salt content, due to neutralization. In addition, dextrose yield was limited to approximately 85% and off-colors and flavors were often a result of the acid hydrolysis process. The change to enzymatic hydrolysis in the 1960s eliminated all the drawbacks of the acid hydrolysis process and increased the yield of dextrose to between 95 and 97%. This chapter will focus on the physical and enzymatic steps required to convert starch to sugars that are utilizable by yeast.

Carbohydrate chemistry: the basics

When most people think of carbohydrates, they think of sugar or starch. Carbohydrates are thus called because they are essentially hydrates of carbon. They are composed of carbon and water and most may be represented by the general formula $(CH_2O)_n$. For example, the empirical formula for D-glucose is $C_6H_{12}O_6$, which can also be written $(CH_2O)_6$. There are four major classes of carbohydrate that are of general interest: monosaccharides, disaccharides, oligosaccharides and polysaccharides. Monosaccharides, or simple sugars, consist of a single polyhydroxy aldehyde or ketone unit. The most abundant monosaccharide in nature is, of course, the 6-carbon sugar D-glucose.

Disaccharides encompass sugars such as sucrose, which is an important transport carbohydrate in plants. Lactose, a disaccharide of glucose and galactose, is commonly called milk sugar. Oligosaccharides (Greek *oligos*, 'few') are made up of short chains of monosaccharide units joined together by covalent bonds. Most oligosaccharides in nature do not occur as free entities but are joined as side chains to polypeptides in glycoproteins and proteoglycans. Polysaccharides consist of long chains of hundreds or even thousands of linked monosaccharide units and, as will be discussed, exist in either linear or branched chain form. Knowledge of the terminology associated with the organizational structure of monosaccharides, such as D-glucose, is fundamental to an understanding of how polysaccharides differ and how polysaccharide-degrading enzymes, such as amylases, function.

Monosaccharide structures are often drawn as open Fischer projection formulae in which the sugar is depicted in either its D- or L-configuration. Most naturally occurring sugars are D-isomers, but D- and L-sugars are mirror images of each other. The D or L designation refers to whether the asymmetric carbon farthest from the aldehyde or keto group points to the right or the left as shown for D-glucose and L-glucose (Figure 2).

The ring or cyclic forms of monosaccharides are most commonly drawn as Haworth projection formulae, as shown for glucose in Figure 3. Although the edge of the ring nearest the reader is usually represented by bold lines, the six-membered pyranose ring is not planar, as Haworth projections suggest. More importantly, however, the numbering system for the carbon atoms in the glucose structure should be carefully noted. Carbon atom number one, also known as the anomeric carbon atom, is the first carbon encountered when going in a clockwise direction from the oxygen atom in the ring structure, and the other carbon atoms, C2 to C6, are numbered as indicated in Figure 3. When the hydroxyl or OH group at the anomeric carbon atom extends *below* the ring structure of D-glucose, the sugar is stated to be in the α (alpha) configuration. When the hydroxyl group at C1 extends *above* the ring, the sugar is in the ß (beta)-configuration. These seemingly minor structural changes represent the major difference between key natural polysaccharides such as starch and cellulose.

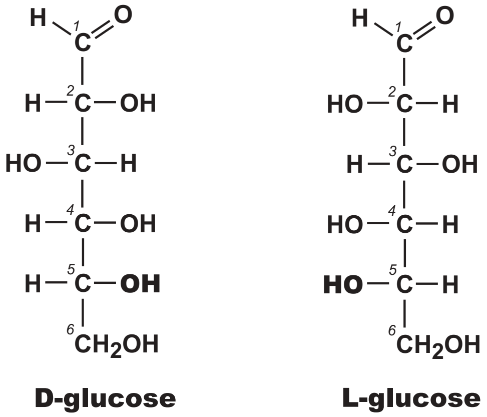


Figure 2. Fischer projection formulae for D- and L- glucose.

STARCH

Native starch is a large polymer made up of D-glucose molecules linked together to form a linear polymer called amylose or a branched polymer called amylopectin (Figure 4).

In amylose, the glucose subunits are linked together by bonds between the anomeric carbon of one subunit and carbon number 4 of the neighboring subunit. Because the OH group at the anomeric carbon is below the apparent plane of the glucose subunit, the glucosidic linkage formed is stated to be in the α-configuration and the bond is termed α(1→4). Amylose chains typically contain between 500 and 2,000 glucose subunits (Lehninger, 1982).

In amylopectin, glucose subunits are also linked in α(1→4) configuration in the linear parts of the polysaccharide but branch points occur approximately once every 25 glucose units where the terminal glucose in a parallel amylose chain is involved in an α(1→6) linkage. These branch points are resistant to hydrolysis by many amylases. Some amylases are totally incapable of hydrolyzing the α(1→6) bonds while others hydrolyze the 1→6 bonds more slowly than the 1→4 bonds.

Glycogen is a very closely related structure to amylopectin and serves as the glucose storage polysaccharide in animals. The main difference is that the branch points in glycogen occur more frequently, approximately once every 12 glucose units. Cellulose is a linear, unbranched homopolysaccharide of 10,000 or more D-glucose units linked by 1→4 glucosidic bonds. It might be expected, therefore, to closely resemble amylose and the linear chains of glycogen, but there is a critical difference: in cellulose the 1→4 linkages are in the ß-configuration (Figure 5). This apparently trivial difference in the structures of starch and cellulose results in polysaccharides having widely different properties. Because of their ß-linkages, the D-glucose chains in cellulose adopt an extended conformation and undergo extensive side by side aggregation into extremely insoluble fibrils. Since no enzyme capable of hydrolyzing cellulose is secreted by the intestinal tract of vertebrates, cellulose cannot easily be

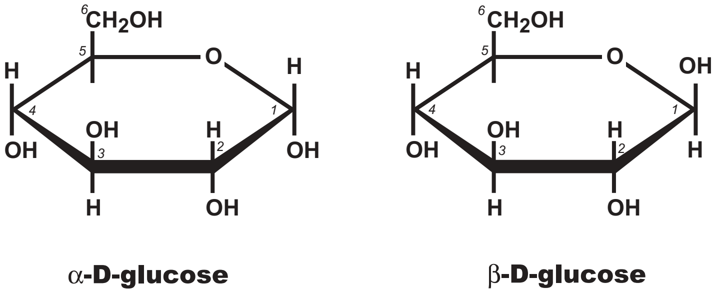


Figure 3. Haworth projections of α-D-glucose and ß-D-glucose.

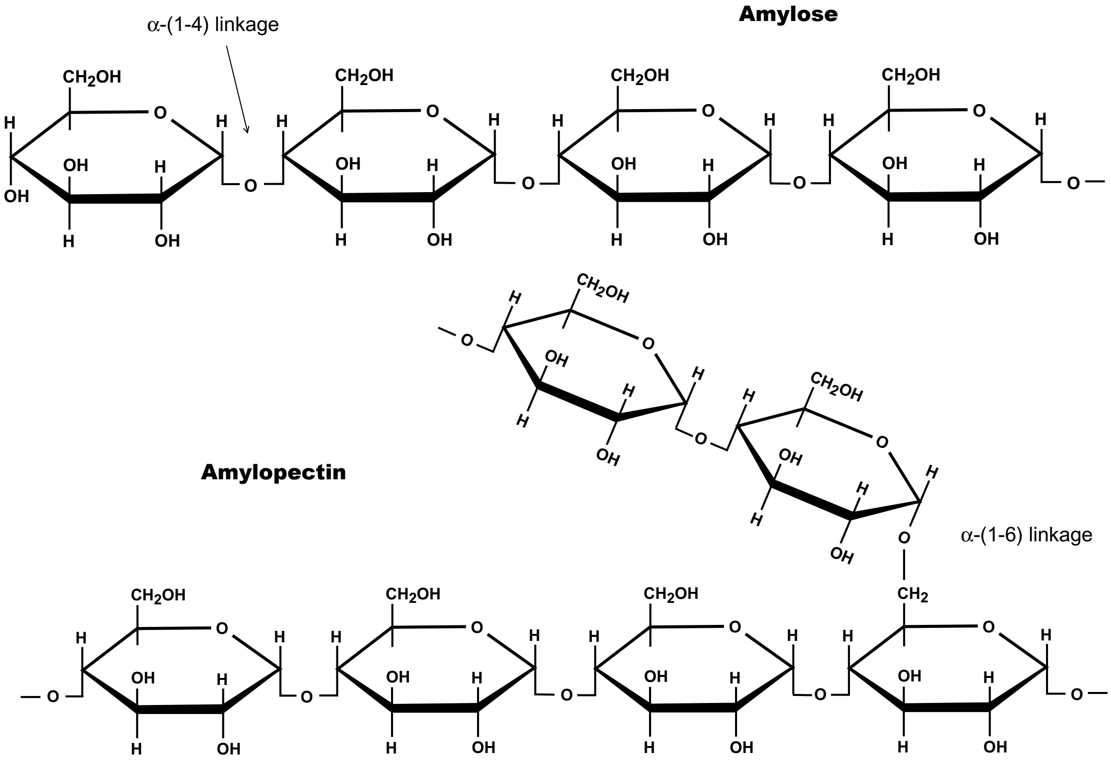


Figure 4. Structure of amylose and amylopectin; the polysaccharides that comprise starch.

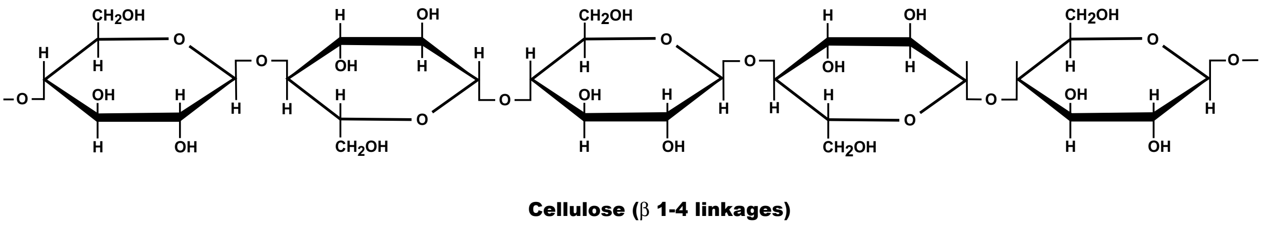


Figure 5. Structure of cellulose showing ß(1→4) linkages between D-glucose units.

digested and its D-glucose units remain unavailable as food to most non-ruminant animals.

In contrast, the α-linkage of starch allows the molecule to be more flexible, taking on a helical configuration in solution which is stabilized by hydrogen bonding. Furthermore, the bottlebrush-like structure of amylopectin lends itself to less densely packed associations between adjacent starch molecules and further enhances water solubility and access by starch-degrading enzymes. The ratio of amylose to amylopectin is a characteristic of each starch source and has an impact on its gelatinization properties (see below). For example, four classes of corn starch exist. Common corn starch contains approximately 25% amylose, while waxy maize is almost totally comprised of amylopectin. The two remaining corn starches are high-amylose varieties with amylose contents ranging from 50 to 75%. Other typical amylose contents are 20% for potato, 17% for tapioca and 25% for wheat. Common rice starch has an amylose:amylopectin ratio of approximately 20:80 while waxy rice starch contains only about 2% amylose (Table 1).

Table 1. Ratio of amylose to amylopectin (percent of total starch) in various starch sources.

Starch source	*Amylose (%)*	*Amylopectin (%)*
Common corn	25	75
Waxy maize	1	99
High amylose corn	50-75	25-50
Potato	20	80
Rice	20	80
Waxy rice	2	98
Tapioca/cassava/manioc	17	83
Wheat	25	75
Sorghum	25	75
Waxy sorghum	<1	>99
Heterowaxy sorghum	<20	>80

In plants, starch occurs in the form of minute, discrete granules which are visible microscopically. Their size varies depending on starch source. At room temperature, starch granules are quite resistant to penetration by both water and hydrolytic enzymes, due to the formation of hydrogen bonds within the same molecule and between neighboring molecules. However, these intra- and inter-molecular hydrogen bonds can be weakened as the temperature is increased. In fact, when an aqueous suspension of starch is heated, the hydrogen bonds rupture, water is absorbed and the starch granules swell. This process is termed *gelatinization* because the resulting solution has a highly viscous, gelatinous consistency. This is the same process long employed to thicken broth in food preparation.

The exact temperature required for gelatinization to occur depends on a variety of factors, including granule size and structure and the aforementioned amylose to amylopectin ratio. For example, tapioca starch has a lower gelatinization temperature than rice starch, although both have similar amylose contents. This is because tapioca starch granules are much larger than rice starch granules and, as a result, swell more easily. Simply put, the larger the granule, the less molecular bonding is present and thus the granules swell faster in the presence of water and elevated temperatures. Waxy corn and common corn starch both have the same granule size but waxy corn will swell to a greater degree and each will gelatinize at different temperatures. This is due to their different amylose:amylopectin ratios. One can visualize that amylose molecules, because of their linear structure, will lie in closer proximity to each other than the brush-like amylopectin molecules. More extensive inter-molecular hydrogen bonding will occur between the closely bunched amylose polymers in high-amylose content starch sources than will occur in high-amylopectin sources. Consequently, it requires more energy to break the hydrogen bonds in high-amylose starch sources, which results in higher gelatinization temperatures.

Table 2. Typical temperature ranges for the gelatinization of various cereal starches.

Cereal starch source	*Gelatinization range (°C)*
Barley	52-59
Wheat	58-64
Rye	57-70
Corn (maize)	62-72
High amylose corn	67->80
Rice	68-77
Sorghum	68-77

Kelsall and Lyons, 1999

Gelatinization must occur in order for the starch-degrading amylase enzymes to begin the process of converting this plant storage polysaccharide to fermentable sugars.

Enzymatic hydrolysis of starch

Although the use of industrial enzymes for starch hydrolysis did not become widespread until the 1960s, Payen and Persoz reported the reactions involved some 130 years earlier (Payen and Persoz, 1833). The enzyme used for the initial dextrinization or liquefaction of starch is *α-amylase*. The more scientifically correct or systematic name for this type of enzyme is 1,4-α-D-glucan glucanohydrolase and it is also described by the following Enzyme Commission (EC) number, as laid down by the International Union of Biochemistry (IUB): 3.2.1.1. This number denotes that it is a hydrolase (3) of the glycosidase subcategory (3.2), which hydrolyzes O- glycosyl linkages (3.2.1). Because it is the first enzyme appearing in this category, its complete IUB number is 3.2.1.1. When faced with the IUB-designated nomenclature for enzymes, the value of even a modicum of knowledge in the area of carbohydrate linkage terminology becomes apparent. For example, in the case of α-amylase, one can tell from its systematic name that it hydrolyzes 1→4 bonds linking α-D-glucose residues. A further characteristic of α-amylases is that they are endo-acting enzymes, meaning that they attack the starch polymer from within the chain of linked glucose residues rather than from the ends. They randomly cleave internal α(1→4) bonds to yield shorter, water-soluble, oligosaccharide chains called *dextrins,* which are also liberated in the α-configuration. α-Amylases are metallo-enzymes and require calcium ions to be present for maximum activity and stability. Finally, α-amylases cannot cleave α(1→6) bonds and bypass the branch points in amylopectin. When this occurs, the residual products are called *α-limit dextrins*.

Commercial sources of α-amylase are produced mainly by *Bacillus* species, for example, *Bacillus amyloliquefaciens* and *B. licheniformis*. Because cooking conditions for starch will be dealt with in detail elsewhere, it is sufficient to point out here that the choice of α-amylase is based principally on tolerance to high temperatures and that this varies quite widely among enzyme sources. One common approach is to cook the starch at approximately 105ºC in the presence of a thermostable α-amylase, followed by a continued liquefaction stage at 90-95ºC. The maximum extent of hydrolysis, or dextrose equivalence (DE), obtainable using bacterial α-amylases is around 40, but care should be exercised not to overdose or prolong treatment since this can lead to the formation of maltulose (4-α-D-glucopyranosyl-D-fructose), which is resistant to hydrolysis by α-amylases and glucoamylases. It must be remembered that the principal function of liquefaction is to reduce the viscosity of the gelatinized starch, thereby rendering it more manageable for subsequent processing.

The general action of amylases on starch is shown in Figure 6, where the first phase of enzymatic starch degradation, i.e. the production of both dextrins and α-limit dextrins from starch, is schematically depicted. As stated earlier, the next stage is termed the true saccharification phase, which under most practical circumstances is effected by a class of amylases called *glucoamylases*. Also included in Figure 6 is a depiction of the mode of action of *pullulanases* (α-dextrin 6-glucanohydrolase; EC No. 3.2.1.41), which belong to a category of starch-degrading enzymes known as debranching enzymes. The action of pullulanases concerns the hydrolysis of 1,6-α-D-glucosidic linkages in amylopectin, glycogen and their nascent limit dextrins, generated by α-amylase activity. The application of dedicated pullulanases in the ethanol industry is not widely encountered, although commercial preparations are available. This is due both to economic considerations and the fact that, in addition to a predominantly exo-acting α(1→4) hydrolytic activity, glucoamylases (see below) can also hydrolyze 1→6 bonds to a limited degree. Given that the use of a glucoamylase component is absolutely required for efficient saccharification to occur, glucoamylase inclusion is normally relied upon to perform the dual functions of an amyloglucosidase and a debranching enzyme. Nevertheless, pullulanases deserve to be mentioned as a category of enzymes in their own right because they are likely to become much more affordable in the coming years.

In the beverage alcohol industry, *ß-amylase* (1,4-α-D-glucan maltohydrolase; 3.2.1.2) is another enzyme encountered in the starch conversion process. Like α-amylase, this enzyme cleaves α(1→4) linkages but attacks starch in an ‘exo’ rather than an ‘endo’ fashion. The enzyme cleaves maltose (a disaccharide of glucose) in a stepwise manner from the non-reducing end of the starch polymer. The enzyme cannot bypass α(1→6) branch points to attack linear 1→4 bonds on the other side and generates *ß-limit dextrins* as a result. Hence, the enzyme is most effective when used in conjunction with a debranching enzyme. ß-amylases are also utilized in the syrup industry for the production of high-maltose syrups from starch (McCleary, 1986). This enzyme represents a good example of the confusion that sometimes arises in enzyme terminology. One could easily be forgiven for questioning how a ß-amylase can act upon α(1→4) linkages. The main reason for this confusion is due to the fact that enzyme nomenclature is based upon the configuration of the released product rather than that of the bond being hydrolyzed. As ß-amylase

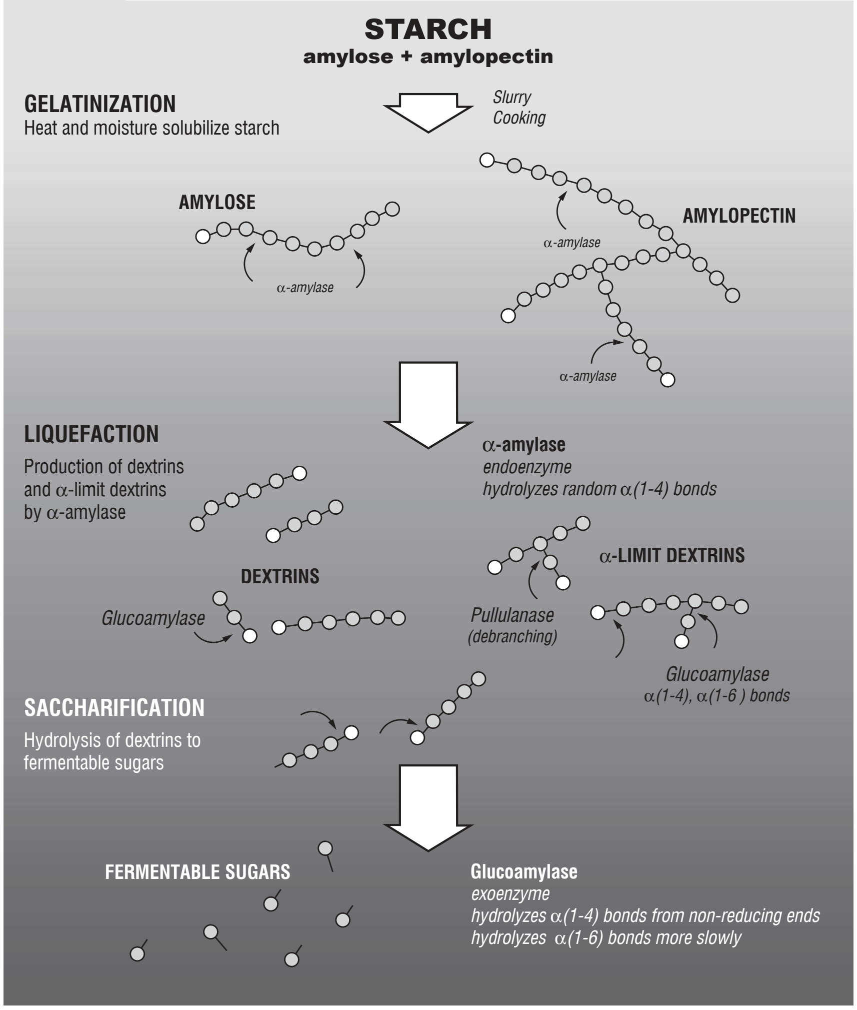

STARCH
amylose + amylopectin
GELATINIZATION
Heat and moisture solubilize starch
Slurry
Cooking
AMYLOSE
α-amylase
AMYLOPECTIN
α-amylase
α-amylase
LIQUEFACTION
Production of dextrins
and α-limit dextrins
by α-amylase
α-amylase
endoenzyme
hydrolyzes random α(1-4) bonds
DEXTRINS
α-LIMIT DEXTRINS
Glucoamylase
Pullulanase
(debranching)
Glucoamylase
α(1-4), α(1-6) bonds
SACCHARIFICATION
Hydrolysis of dextrins to
fermentable sugars
FERMENTABLE SUGARS
Glucoamylase
exoenzyme
hydrolyzes α(1-4) bonds from non-reducing ends
hydrolyzes α(1-6) bonds more slowly

Figure 6. Schematic representation of the action of amylases on starch.

catalyzes the removal of maltose units from starch, it produces what is known as a Walden inversion of the OH group at C-1, giving rise to *ß-maltose* (Belitz and Grosch, 1999). Thus, the products of bacterial and fungal α-amylases are all in the α-configuration, while the products of ß-amylases exist in the ß-configuration.

As mentioned, the true saccharification or conversion of dextrins to glucose is conducted, in the vast majority of cases, by glucoamylase**,** although other enzymes, including some α-amylases, have significant saccharifying activity in their own right. Glucoamylase is also known as *amyloglucosidase* (glucan 1,4 –α-glucosidase; EC No. 3.2.1.3) and its main activity is the hydrolysis of terminal 1,4-linked α-D-glucose residues successively from the non-reducing ends of dextrins causing the release of ß-D-glucose. In other words, it is an exo-acting enzyme that cleaves single molecules of glucose in a stepwise manner from one end of the starch molecule or dextrin. Reference has also been made to the fact that glucoamylases can hydrolyze α(1→6) bonds to a certain extent. However, these branches are cleaved at a rate approximately 20 to 30 times slower than the cleavage of α(1→4) bonds by the enzyme. Extra glucoamylase can be added to compensate for this slower rate but this can cause undesirable side reactions to take place, whereby glucose molecules repolymerise in a reaction termed reversion, forming isomaltose and causing a decrease in glucose yield.

Glucoamylases are isolated from fungal

sources such as *Aspergillus niger* and *Rhizopus sp*. Fungal enzymes, by nature, are less thermotolerant than their bacterial counterparts and therefore the temperature maxima for glucoamylases tend to be in the region of 60ºC while their pH optima normally lie between pH 4.0 and 4.5. These conditions of temperature and pH can cause significant problems in plants using dedicated saccharification tanks because they are quite conducive to the growth of certain bacterial contaminants. The claimed benefit of pre-saccharification of mash prior to fermentation is that a high level of glucose is made available at the start of fermentation. However, in addition to infection problems, such an approach can lead to the generation of non-fermentable reversion products, such as isomaltose.

Because of the drawbacks of mash pre-saccharification, a popular approach is to saccharify simultaneously with fermentation (SSF or **S**imultaneous **S**accharification and **F**ermentation). The requirement for high glucose concentrations at the start of fermentation is met by the addition of glucoamylase during the filling of the fermentor. Although the concern with such a system is that glucose may become limiting during the fermentation, this is not a problem in practice and is merely a case of metering in the correct amount of enzyme. Glucose is used up as it is produced and thus there is little risk of reversion reactions occurring. An additional attraction to this approach is that it lends itself to the use of somewhat non-conventional enzymes, which can boost ethanol yield by liberating fermentable sugars from non-starch polysaccharide sources. For example, multi-enzyme complexes isolated from fungal cultures grown on fiber-rich, semi-solid or surface culture systems, contain a mixture of amylolytic, cellulolytic and other polysaccharidase activities which have temperature and pH optima that quite closely match those found in distillery fermentors. One commercial preparation, Rhizozyme™, is a surface culture enzyme gaining increasing use in the fuel ethanol industry. These enzymes satisfy the requirement to saccharify dextrins to glucose but have the additional activities required to attack, for example, ß-linked glucose polymers, if present.

Summary

The objective of industrial alcohol production is to produce the highest ethanol yield possible. This requires the greatest attainable conversion of starch to fermentable sugars because yeast cannot use starch in its native state. The use of starch-degrading enzymes represented one of the first large-scale industrial applications of microbial enzymes. Traditionally, two main enzymes catalyze the conversion of starch to glucose. In the first stage of starch conversion, α-amylase randomly cleaves the large α-1,4-linked glucose polymers into shorter oligosaccharides at a high reaction temperature. This phase is called liquefaction and is carried out by bacterial enzymes. In the next stage,

Table 3. Enzymes used in the starch hydrolysis process.

Enzyme	*EC number*	*Source*	*Action*
α-amylase	3.2.1.1	*Bacillus sp.*	Only α-1,4-linkages are cleaved to produce α-dextrins, maltose and oligosaccharides G(3) or higher
		Aspergillus oryzae, A. niger	Only α-1,4-linkages are cleaved producing α-dextrins, maltose and G(3) oligosaccharides
α-amylase (saccharifying)	3.2.1.1	*Bacillus amylosaccharlticus*	Cleaves α-1,4-linkages yielding α-dextrins, maltose, G(3), G(4) and up to 50% (w/w) glucose.
ß-amylase	3.2.1.2	Malted barley	α-1,4-linkages cleaved, from non-reducing ends, to yield limit dextrins and ß-maltose.
Glucoamylase	3.2.1.3	*Aspergillus sp. Rhizopus sp.*	α-1,4 and α-1,6-linkages are cleaved, from the non-reducing ends, to yield ß-glucose.
Pullulanase	3.2.1.41	*B. acidopullulyticus*	Only α-1,6-linkages are cleaved to yield straight chain dextrins.

called saccharification, glucoamylase hydrolyzes the oligosaccharides or dextrins into free glucose subunits. Fungal enzymes are normally employed to effect saccharification and these operate at a lower pH and temperature range than α-amylase. Depending on the industry and process involved, one sometimes encounters additional enzymes, such as pullulanase, which is added as a debranching enzyme to improve the glucose yield.

Despite the intricate nature of enzyme nomenclature and the rather strict reaction conditions which must be adhered to in order to achieve maximal enzyme performance, their purpose is very simple: to provide fuel to yeast for the biosynthesis of ethanol. This goal must not be lost sight of and new approaches, enzymatic or otherwise, need to be explored in an effort to liberate additional fermentable sugars from common cereal sources. The application of surface culture and fungi that produce multi-enzyme complexes, encompassing several polysaccharidase activities in addition to the requisite, traditional, amylolytic activities, represents one promising approach towards maximizing the release of utilizable carbohydrate from both starch- and non-starch-based cereal polysaccharides.

References

Belitz, H.-D. and W. Grosch. 1999. *Food Chemistry*. (M.M. Burghagen, D. Hadziyev, P. Hessel, S. Jordan and C. Sprinz, eds). Springer-Verlag, Berlin Heidelberg. pp. 237-318.

Kelsall, D.R. and T.P. Lyons. 1999. Grain dry milling and cooking for alcohol production: designing for 23% ethanol and maximum yield. In: *The Alcohol Textbook*, 3rd ed.(K.A. Jacques, T.P. Lyons and D.R. Kelsall, eds). Nottingham University Press, UK. pp. 7-24.

Lehninger, A.L. 1982. *Principles of Biochemistry*. (S. Anderson and J. Fox, eds). Worth Publishers Inc. New York. pp. 277-298.

McAloon, A., Taylor, F., Yee, W. Ibsen, K. and R. Wooley. 2000. Determining the cost of producing ethanol from corn starch and lignocellulosic feedstocks: A Joint Study Sponsored by U.S. Department of Agriculture and U.S. Department of Energy. National Renewable Energy Laboratory Technical Report/ TP-580-28893.

McCleary, B.V. 1986. Enzymatic modification of plant polysaccharides. Int. J. Biol. Macromol. 8:349-354.

Payen, A. and J.F. Persoz. 1833. Mémoire sur la diastase, les principaux produits de ses réactions, et leurs applications aux arts industriels. Ann. Chim. Phys. 53: 73-92.

Chapter 4

Grain handling: a critical aspect of distillery operation

DAVID J. RADZANOWSKI

Alltech Inc., Nicholasville, Kentucky, USA

Introduction

Until recently, sanitation in grain handling has been basically ignored by the distilling industry, except for those engaged in beverage alcohol production. Now that we have begun to discuss and accept the 'Biorefinery' concept, we are forced to realize that a good sanitation program and procedures are essential to the bottom line. Outbreaks of food animal diseases such as BSE and food-borne diseases such salmonella, as well as prohibitions against mycotoxins and insecticides in grains going into food animals and ultimately the human food chain, have grabbed our attention. We suddenly realize that distillers dried grains with solubles (DDGS) is a valuable co-product and not just an unavoidable nuisance. Also, if we cannot sell it because of contamination, we are out of business.

We first became aware of the importance of a sanitation program in grain handling systems some years ago in the brewing industry. Breweries by definition prepare a food product, beer, and are subject to all possible scrutiny by the Food and Drug Administration (FDA) and related state and local food safety bureaus. Breweries routinely underwent very involved inspections for food contamination and looked forward to them with much dread. In the 1970s, a group of federal inspectors appeared at one plant and after providing the proper credentials and the warrants necessary for the inspection, surprised the staff by asking only for a sample of brewer's spent grains. The staff relaxed and gladly obtained a sample of spent grains under the inspectors' directions.

Two weeks later the plant was upset with a notice of citation for improper sanitation procedures since insect fragments, mold, mycotoxins and insecticide residuals were found in those spent grains. Since animal feed laws were less stringent in the 1970s than today, the plant was allowed to continue selling the spent grains. The agency just used the contamination detected as a means of bringing attention to an inadequate sanitation program as established for breweries under the Federal Food, Drug and Cosmetic Act.

Good manufacturing practices

Since 1969, the Act has contained guidelines called the Good Manufacturing Practices or the GMPs (FDA, 2000). This Amendment to the Act is titled 'Human Foods, Current Good Manufacturing Practice (Sanitation) in Manufacture, Processing, Packing or Holding'. Sections 3 through 8 of the amendment apply in determining whether the facilities, methods, practices and controls used in the manufacture, processing, packing or holding of food are in conformance with and are operated or administered in conformity with good manufacturing practices to produce under sanitary conditions, food for human consumption.

Knowledge of the GMPs, with an eye toward meeting the outlined standards, is a valuable guide toward setting up an operation that facilitates the proper handling of grain. Section 3 of the amendment provides guidelines for the correct positioning of the plant and the care of the grounds. It goes further to detail the appropriate construction of the facility utilizing cleanable surfaces, adequate lighting, ventilation, employee facilities and screening and protection from pests. Section 4 contains the requirements for the equipment and utensils. All must be suitable for the intended use, durable, cleanable and kept in good repair.

Sections 5 and 6 are closely related. They deal with the needs for adequate water, plumbing and sewage disposal, toilet and hand washing facilities for employees, and the proper disposal of offal and rubbish. The requirements for general maintenance, pest control and sanitizing of equipment and utensils are described in detail.

To ensure that food will not be contaminated, Section 7 requires the inspection of all raw materials and ingredients as well as the inspection of the containers and the common carriers delivering the goods. This section also requires frequent cleaning and sanitization, processing under conditions to minimize bacterial and micro-organic growth, chemical and biological testing and the maintenance and retention of records.

Under Section 8, plant management is required to assure that employees are free from disease, observe good cleanliness practices in clothing as well as personal habits and that they are properly trained and educated to perform their duties in a safe and sanitary manner.

STRICTER REQUIREMENTS LIKELY

Distillers in the United States, as well as in other countries, do not normally come under the jurisdiction of the FDA or related organizations. They are normally governed by bureaus like the US Bureau of Alcohol, Tobacco and Firearms (BATF), a regulatory agency that imposes and collects tax monies on the ethanol generated by a distiller. Recent changes in the US with the creation of the Homeland Security Act have split up the BATF and reduced the strength of the organization pertaining to alcohol producer enforcement. This leaves a gap that the FDA seems to be filling, slowly but forcefully. What does this mean to the distiller? In all probability, stricter requirements demanding reduced contamination in co-products in animal feeds such as DDGS will be established. We have already seen some activity from other countries rejecting DDGS generated in the US due to the content of mycotoxins and other unwanted contamination. How can we help prevent rejection or seizure of this valuable by-product? Adopting strict sanitation programs is an aid to designing or remodeling plants to conform to the guidelines needed.

Where do we start?

William Schoenherr, formerly of the Lauhoff Grain Company, which is now part of Bunge Milling is considered the father of sanitation in grain handling and storage facilities as well as in breweries. Schoenherr started as an FDA inspector and brought his enormous expertise to the grain industry. Lauhoff recognized that it had obtained a great asset and allowed Bill to share his expertise with the grain handling world and particularly their customers. Schoenherr always talked about a number of key factors that must be considered in a good sanitation program. We will use these as guidelines for the following discussion.

FORMS OF CONTAMINANTS, DETECTION AND REMOVAL

First, it is important to be aware of the different forms of contamination that may be present in the raw materials brought in for processing. Second, everything possible must be done to ensure detection of such contamination before it enters the facility. And, third, everything possible must be done to avoid, remove or destroy the contaminants before using these raw materials. These three are very closely related.

What kind of contaminants can we expect?

Among others, insects, rodents and birds and their droppings, stone, cobs, weed seeds, glass, wood, rags, tramp metal, residual insecticides and fumigants, water, bacteria, mold and mycotoxins. Some of these are from unclean grain handling and transport. Others have been added as fillers, adding weight without producing usable substrate. Some, like stone and tramp metal, are dangerous and may cause fires and explosions in the grain handling system. The more difficult contaminants to detect such as residual insecticides and fumigants as well as mycotoxins, can be present at levels that can result in unmarketable DDGS.

Most operations are located close to the source of the substrate used for fermentation, so most of the material is received in trucks with only a limited amount of rail transit. Samples are generally obtained by probing each truck in two places, front and back, and then composited. Statistically, this is not sufficient. Two pounds of sample from a 50,000-pound load is not very indicative of the condition of the contents of the truck.

Bushel weight, moisture, damaged kernel and broken kernel/foreign material tests are run immediately. High insect infestation may be determined by visual inspection, but low infestations may be more difficult to find. Also, the true grain weevils, because they bore into kernels, may not be visible on the surface of the grain. Only a few plants use a black light to detect aflatoxin presence. Black light is also usable for the detection of rodent urine contamination. This should be a routine test for all shipments in all plants. NIR technology offers a solution to many of the grain testing needs for quick decision making by distillers. That subject is covered in a separate chapter in this volume.

Do not neglect the senses. Examine the grain, checking for insect presence, rodent droppings, bird feathers as well as foreign seeds and extraneous matter such as cobs, straw, wood and metal. Smell the material for any hint of mold presence or chemical contamination. Feel the grain for moisture and slime presence.

Most plants use the specifications for No. 2 corn as a guideline, which call for a bushel weight of 56 pounds, a limit of 0.2% heat damaged kernels, 5% total damaged kernels and 3% total broken kernel/foreign material presence. A recent discussion with a group of farmers supplying corn to some of the newest fuel ethanol plants coming on stream was very revealing concerning the state of the corn market in some areas of the country. The farmers were quite happy that the new distillers were paying the same price for No. 2, 3, 4 and 5 grain as they are for No. 1. This seems not only wasteful on the part of the distiller, but potentially dangerous. The more allowable extraneous content in the lower grade incoming grain, the greater the contribution to lower yields and the higher likelihood of gross contamination and possible ignition of fires and explosions.

High moisture is the most common reason for rejection of a shipment. This is a good start. High moisture contributes to difficulties in milling and handling and to lower yields. High moisture also contributes to elevated mold growth, which typically leads to contamination with mycotoxins that become concentrated in the DDGS.

First, decide on specifications for acceptance of the incoming substrate. Next, provide the people and the testing equipment to determine if the substrate meets those specifications. Stick to the established specifications. Only when satisfied should you begin to unload the material into the plant's infrastructure.

RECEIPT AND STORAGE

Specifications should call for all shipments to be received in covered hopper railroad cars and hopper trucks fully covered by tarpaulin or some other means. This will help prevent appearance of rodents and other pests while the shipment is in transit.

Once the shipment is accepted, it can then proceed to the unloading area. Most people drop the contents of the railcar or truck into a pit from which the material is conveyed in some manner to the storage bin. It is important that this station be constructed in a manner that allows it to be kept in good sanitary condition without much effort. Pay attention to the grid covering the pit. Some grids have wide-spaced bars that could allow whole rats to pass through to the pit and onward to the bin. This is unacceptable. The grid system should be designed for the rapid flow of the grain and nothing else.

There are many different styles of grain conveying systems, including bucket elevators, positive and negative pneumatic systems, belt conveyors and the newer pulse pneumatic systems. The most user unfriendly and dangerous system seems to be the bucket elevator. This is due to the high number of parts with a greater maintenance requirement and the higher number of possible ignition points. Manufacturers have attempted to alleviate some of this by the introduction of plastic buckets, but the hangers and chains are still, for the most part, made of metal. Bucket elevators also provide more harborages for insects and are harder to clean and fumigate. Pneumatic systems tend to be abraded by the impingement of the grain on the tubing system, but a well-designed and balanced system can extend the life expectancy of the tubes indefinitely.

Grain should ideally be cleaned on the way to the bin. Why store dust, straw, cobs, wood, metal, dead rats and other extraneous material? Unfortunately, most of the systems we see are similar to Figure 1.

The facility illustrated in Figure 1 is typical in breweries since the brewers are not really receiving raw materials, but barley that has been cleaned and processed by malting. For brewers such a system works well. The earlier fuel ethanol plants had systems like this installed because the engineers had been designing for breweries for a number of years and did not recognize that distillers are involved in handling a different type of material. Distillers have become aware of this and are requesting that cleaning systems be placed ahead of the bins.

Keep records of the amount of debris removed, as you do not want to pay for trash. Keep a record of the net amount of clean substrate entering the storage bin. This figure will be helpful later in determining true yield.

Bins should be constructed of concrete or metal and be well sealed. We have seen bins constructed of corrugated metal sheets that leaked so badly that the grain was beginning to ferment in the storage bin after a very light rainfall. One of the most important design criteria for grain bins is their ability to be fully emptied.

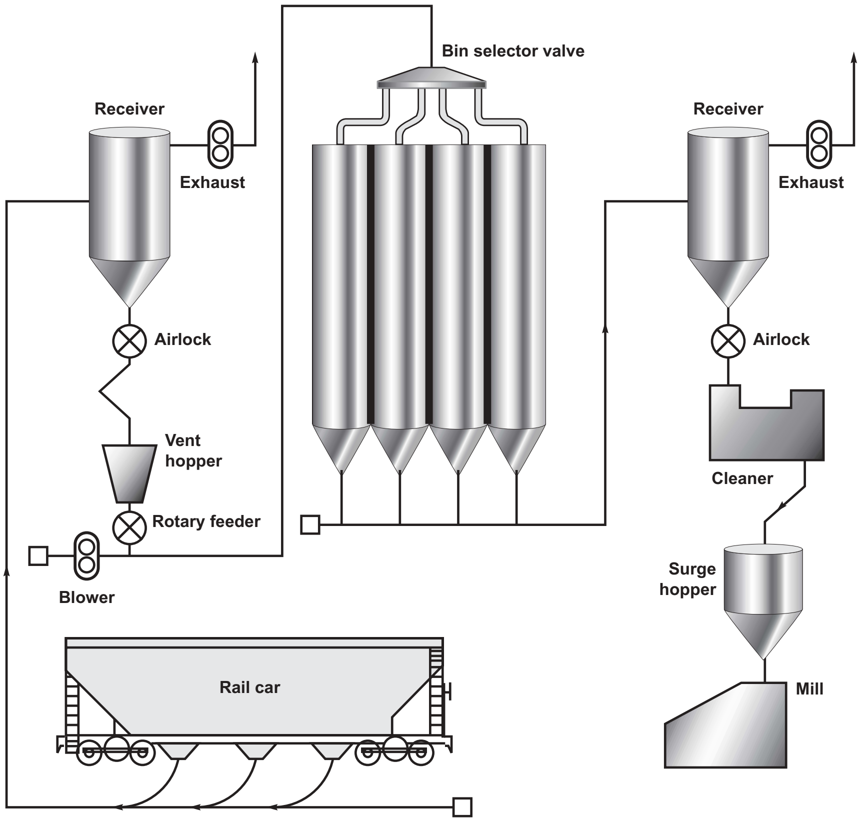


Figure 1. Typical configuration of a grain storage receiving system.

We have seen too many storage bins at distilleries that hold 3-5 days of material that cannot be totally emptied. This can be very dangerous and conducive to infestation. Mold growing in stationary grain simply contaminates successive loads.

One of the great tools for protection against insect infestation is the understanding of the life cycles of the various insect pests. Interrupting that life cycle prevents an infestation from becoming established. A 3-5 day storage limit of grain in a bin will interrupt the life cycle of the common grain insects, but the bin must be totally emptied each time it is filled. If the design does not allow total emptying, there will be a continuous infestation.

STORAGE CAPACITY

How large should storage capacity be? Financial and logistic experts must contribute to this decision. Storage capacity must accommodate both the processing schedule and cash flow consideration. If running 24-hrs a day, seven days a week and 50 weeks of the year, then either storage capacity or local sources of substrate must keep the plant supplied. Local supplies reduce storage needs, so that capacity may be needed only to maintain supplies through weekends and holidays. If shipments must come from afar, more capacity is needed. This is where the logistics advice is needed.

The sanitation program

Sanitation programs must be adjusted to the type of facility. Very few large operations today are contained within a structure. Most are of an open air design. The enclosed plant has the sanitation advantage of controlled entry and egress, making it easier to exclude rodents and birds. Regardless of design, full inspection and cleaning techniques must not be relaxed. Spills must be cleaned up quickly to prevent attracting pests. Scrape, shovel and vacuum the debris. Never use an air hose since the subsequent dispersal just spreads infestation and infection.

Any inspection and cleaning program should be familiar with storage insect life cycles and take advantage of this knowledge. Full inspections of the total operation should be made every two weeks and inspections of the more critical zones should take place on a weekly basis. Any evidence of an unwanted invasion should be dealt with immediately. The cleaning schedule should also be devised with the insect life cycle in mind. Certain areas of the plant, like the receiving station and subsequent conveyors, dust collectors and air conveyor systems require more frequent inspection, cleaning and possibly fumigation on a very regular basis. The program should determine the risk and set the appropriate schedule.

A pest control program can be dangerous in the hands of unqualified people. Bait stations, insecticides, and fumigants are poisons and harmful to humans, animals and pets as well as the pests they are intended to control. These compounds are handled and applied by licensed individuals, however it is necessary for the person in the company in charge of the sanitation program to have training and knowledge of pest control operations in order to evaluate the work of the hired contractors.

With the direction and agreement of management, establish the program. Determine the inspection schedule. Hire the pest control firm. Determine the schedule for application of the tools and chemicals of the pest control operation. Determine the cleaning schedule. Keep complete records. Record keeping cannot be stressed too strongly. Obtain all reports from the contract pest control operation including the dates of application, the places of application, the chemicals used in the application, the strength of the materials used, the technician performing the service and the records and results of their inspections as well as the results of the service. Review the work of the hired contractor for effectiveness. Review the program and make appropriate changes as they are necessary.

MILLING

When grain leaves the bin on the way to the mill, it should pass through de-stoners to protect the mills from damage and magnets to prevent sparks and explosions. Dry milling operations should also incorporate explosion dampers in the system. This brings us to the question of what type of a mill to use.

Brewers and Scotch or Irish whisky distillers can work very well with a roller mill, preferably

Table 1. Recommended malt sieve analysis for lauter tun and mash filter wort separation systems.

Fraction	*US sieve number (inches)*	*For lauter tun (% retained)*	*For mash filter (% retained)*
Husks I	10 (0.0787)	14	6
Husks II	14 (0.0555)	20	10
Coarse grits I	18 (0.0394)	29	6
Coarse grits II	30 (0.0234)	18	18
Fine grits	60 (0.0098)	9	43
Coarse flour	100 (0.0059)	4	10
Powder	Bottom	6	7

Adapted from ASBC, 1996

with five or six rolls. The roller mills work extremely well with the soft, already modified and malted barley, which is the principal substrate of these operations. Simple adjustments can be made in the grind according to the needs of the wort separation systems in use, whether they incorporate mash filters or lauter tuns.

Table 1 contains the recommended malt sieve analysis for the different wort separation systems. This is based on the American Society of Brewing Chemists (ASBC) testing sieve system with a 100 g (0.22 lb) sample.

For most grains such as corn and other substrates such as cassava chips, hammer mills will probably perform the most efficiently. The proper screen size to use in the US would be number 6 through 8. This is the equivalent of openings in the range of 1/8 to 3/16 inches. In Europe, the screens would be 2 to 4 mm slot size.

A sieve analysis should be accomplished daily (Table 2). The importance of a fine grind cannot be overemphasized, however a very fine grind must be accompanied by good mixing agitation to avoid formation of pills and lumps, creating insufficient exposure of the solids to the liquid.

A fine grind exposes more surface area, facilitating better slurry, cook and liquefying operations. The more surface area exposed, the greater the effectiveness of the enzymes allowing for greater activity and more complete reactions. An idea of the importance of particle size in determining surface area exposure can be obtained by reviewing Table 3, which uses the explanation of the need for small bubbles for better aeration as an example.

Table 2. Typical sieve analysis of corn meal.

Screen size	*Hole size (inches)*	*Corn on screen (%)*
12	0.0661	3.0
16	0.0469	8.0
20	0.0331	36.0
30	0.0234	20.0
40	0.0165	14.0
60	0.0098	12.0
Through 60		7.0

Most distillers weigh the grist on the way to the cooking operation by the use of weigh hoppers and weigh belts. They use these figures for proportioning solids and liquid in the slurrying operation.

Table 3. The free surface area of one inch of air when split up into bubbles of varying size[1].

Bubble diameter (In.)	*Volume of bubble $V=0.5236\ d^3$ (cubic inches)*	*Surface area of bubble $A=3.1416\ d^2$ (square inches)*	*Surface area of 1 cubic inch of air = A/V (square inches)*
1.0	0.5236	3.1416	6
0.5	0.0645	0.7854	12
0.1	0.0005236	0.031416	60
0.01	0.0000005236	0.00031416	600
0.001	-	-	6,000

[1] Note: $\frac{A}{V} = \frac{3.1416\ d^2}{0.5236\ d^3} = \frac{6}{d}$ = surface area

Other considerations

Full attention must be paid to sanitation in all other phases of the operation. Sanitary design of equipment and piping systems is essential. The positioning of sample ports can be conducive to keeping a clean workspace. Containing spills from sample ports by placing buckets to catch the spills is a very commendable practice, but buckets must be emptied and cleaned regularly. Spills from sample ports or repair work should be cleaned up immediately. Besides attracting pests, they also provide good growth media for the bacteria and wild yeast species that cause common distillery infections. These can be easily transferred to the vessels.

As mentioned above, open air plants have the most difficulty in the control of pests since there is no barrier to help prevent the intrusion of the insects, birds and rodents including bats. Besides a good sanitation program, it is also important that any evidence of droppings, feathers and fragments be cleaned up immediately. The Food, Drug and Cosmetic Act standards will require preventing the possibility of product contamination.

Storage areas for DDGS must also be evaluated from a sanitation perspective. Many distillers store DDGS on the ground in covered and sometimes uncovered areas. These areas are totally open to attack by insects, birds, bats, rodents, dogs, cats, raccoons, possum and other animals. The possibility of mold and mycotoxin formation in these areas can also be devastating to the acceptance of the DDGS in the world feed market, which is increasingly recognized as a part of the human food chain.

References

A Guide to Good Manufacturing Practices for the Food Industry. 1972. Lauhoff Grain Company, Danville, IL, USA.

ASBC. 1996. Methods of Analysis (8th Rev. Ed). American Society of Brewing Chemists. ST Paul, MN, USA.

FDA 2000. Good Manufacturing Practices. 21 CFR 110.

The Alcohol Textbook: A reference for the beverage, fuel and industrial alcohol industries (3rd Ed). 1999. (K.A. Jacques, T.P. Lyons and D.R. Kelsall, eds.) Nottingham University Press, UK.

The Practical Brewer: A Master Brewers Association of the Americas. 1999. Masters Brewers Assoc. Amer., Wauwatosa, WI, USA.

Substrates for ethanol production

Chapter 5

Lignocellulosics to ethanol: meeting ethanol demand in the future

CHARLES A. ABBAS

Director of Yeast and Renewables Research, Archer Daniels Midland, Decatur, Illinois, USA

Introduction

The US alcohol industry has the capacity to process about 1 billion bushels of corn per annum; which accounts for most of the fuel ethanol produced domestically. This represents approximately 10% of the expected 2003 US harvest. As production increases to the expected five billion gallons, the amount of corn used for ethanol will also double. Can we continue to use increasing amounts of grain? What would happen if all of US gasoline demand (~150 billion gallons) was replaced with ethanol? (This happened in Brazil in the 1970s and 1980s.) Assuming a harvest of 10 billion bushels (250 million tonnes) and excellent conversion efficiency, the amount of ethanol produced would still fall far short of the target.

Demand for ethanol is increasing at a rate that will require serious consideration of alternatives to the primarily starch-based feedstocks over the next decade. Various lignocellulosic biomass sources such as agricultural residues, wood and forest wastes, municipal solid wastes and wastes from the pulp and paper industry have the potential to serve as low cost and abundant feedstocks for ethanol production (Bothast and Saha, 1997; Galbe and Zacchi, 2002; Katzen *et al.*, 1999; Monceaux and Madson, 1999; Rogers, 1997; Rogers *et al.*, 1997; Singh and Mishra, 1995; Tolan, 1999; von Sivers and Zacchi, 1996). According to a recent US Department of Energy (DOE) draft report, the total lignocellulosic feedstock potentially available in the US is estimated at 623 million dry tons (Figure 1). Excluding the biomass contribution from energy crops and from sludge and biogas, the remaining lignocellulosics that are primarily derived from agricultural crop residues, forest residues and municipal-source wood/paper/organics can be estimated at a total of about 400 million dry tons. Assuming that these available lignocellulosics are converted to ethanol with yields approaching 80 gallons per ton, domestic ethanol production can be expanded further by an additional estimated 32 billion gallons. This figure represents up to 20% of the current US gasoline consumption, which is estimated in 2003 at 150 billion gallons. This chapter serves to provide an overview of progress of research on lignocellulosics and the opportunities and technical challenges to the production of ethanol from these renewable biomass feedstocks.

Major components of lignocellulosics

The composition of lignocellulosics varies greatly among the major categories of plant sources (i.e. softwoods, hardwoods, straws) and

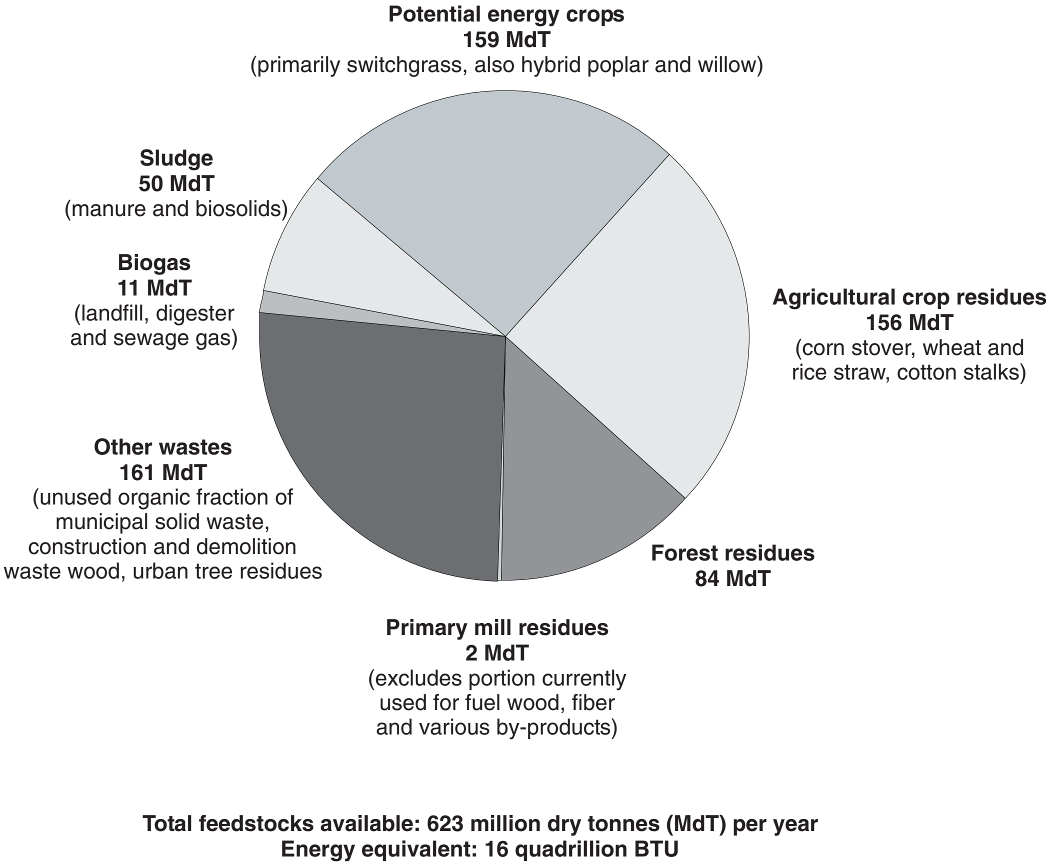


Figure 1. Feedstocks available in the US by biomass type (from: Biobased Products and Bioenergy Roadmap, July 2001 draft).

also varies within each category (Tables 1 and 2). In spite of these differences, the major components of all lignocellulosics are cellulose, hemicellulose, lignin and extraneous materials (Singh and Mishra, 1995). Of these, cellulose is by far the most abundant organic substrate. Cellulose is a linear homopolymer of anhydroglucose residues that are linked via ß-O-1, 4-glycosidic bonds. Different forms of cellulose exist with varying degrees of polymerization and molecular weight (Singh and Mishra, 1995). These forms include a highly crystalline form, which results from the packing of cellulose microfibrils into highly ordered fibers, as well as less ordered and amorphous forms (Lynd *et al.*, 2002). These variations in the crystal structure of cellulose affect its hydrolysis by the action of enzymes and/or chemicals. It is generally recognized that the more ordered or crystalline the cellulose, the less soluble and less degradable it is. Unlike cellulose, hemicellulose consists of shorter heteropoly saccharide chains composed of mixed pentosans and hexosans, which are more readily soluble and consequently amenable to enzymatic degradation. D-xylose and L-arabinose are the major constituents of the xylan or arabinoxylan backbone of the hemicellulose of straws with D-glucose, D-glucuronic, D-mannose and D-galactose as the other primary constituents of the side chains. The principal component of hardwood hemicellulose is glucuroxylan; that of softwoods, glucomannan (Jeffries *et al.*, 1994). Minor constituents, which are ether or ester-linked methyl, acetyl, or acyl groups are also commonly encountered. The D-xylose in the xylan backbone is linked via 1,4-ß-O-glycosidic linkages. The hexosan, araban and other minor constituents of hemicellulose are readily hydrolyzed from the backbone by chemical means, enzymatic means, or a combination of the two. By comparison, the enzymatic hydrolysis of the xylan backbone proceeds at a much slower rate in the presence of these constituents. The considerable variation that is present in the hemicellulose among plant sources is responsible for the great differences in the activity of commercial xylanase or hemicellulases on lignocellulosics.

Table 1. Distribution of cellulose and hemicellulose in wood and straw.

Substrate	*Xylan*	*Araban*	*Galactan* %	*Mannan*	*Glucan*
Hardwood	17.4	0.5	0.8	2.5	50.1
Softwood	5.7	1.0	1.4	11.2	46.3
Straw	16.2	2.5	1.2	1.1	36.5

Table 2. Composition (%) of common lignocellulosic substrates.

Substrate	*Hexosans*	*Pentosans*	*Lignin*
Bagasse	33	30	29
Birch	40	33	21
Corn stover	42	39	14
Groundnut shells	38	36	16
Oat straw	41	16	11
Pine	41	10	27
Rice straw	32	24	13
Sawdust	55	14	21
Wheat straw	30	24	18

The composition of lignin and extraneous materials in plant sources varies considerably (Singh and Mishra, 1995). Lignins are generally distributed with hemicelluloses in the spaces of intracellular microfibrils in primary and secondary cell walls and in the middle lamellae as cementing components to connect cells and harden the cell walls of the xylem tissues (Higuchi, 1990). Lignins have been generally classified into three major groups based on the chemical structure of the monomer units. The groups are softwood lignin, hardwood lignin and grass lignin (Higuchi, 1990). Because the lignin and hemicellulose constituents differ, the cross-links between these polymers vary from plant to plant and tissue to tissue (Jeffries *et al.*, 1994). The primary function of lignin in lignocellulosics is to provide plants with structural rigidity and it also serves as a waterproofing agent in woody tissue (Singh and Mishra, 1995). Lignin is an amorphous noncrystalline polymer composed of highly branched polymeric molecules of phenylpropane-based monomeric units that are linked by different types of bonds, which include alkyl-aryl, alkyl-alkyl and aryl-aryl ether bonds some of which are not readily hydrolyzable (Singh and Mishra, 1995). Thus, lignin is considerably resistant to microbial degradation in comparison to polysaccharides and other natural polymers (Ericksson, 1981; Jeffries *et al.*, 1994; Odier and Artaud, 1992). Lignin is acid stable and insoluble in water but can be solubilized under alkaline conditions. In lignocellulosics the lignin is found closely bound to cellulose and hemicellulose and as a result cannot be readily separated from these carbohydrate polymers using conventional methods (Jeffries *et al.*, 1994; Singh and Mishra, 1995). In lignocellulosics the association of lignin with cellulose and hemicellulose impedes enzymatic degradation of these carbohydrate polymers. For this reason, physical or chemical pretreatments have been designed to disrupt the lignin-carbohydrate matrix to obtain good enzymatic accessibility.

Extraneous materials in lignocellulosics consist of extractable and nonextractable organics such as terpenes, phenols and resins, which include oils, alcohols, tannins, alkaloids, gums, silicate, oxalates and proteins. In some straws and hulls nonextractables such as silica can account for over 10% of the dry weight (Singh and Mishra, 1995).

Processing of lignocellulosics

Processing of lignocellulosics is an essential step in releasing the carbohydrate components in order to derive fermentable feedstocks for the production of ethanol. This goal can be accomplished by combining pretreatment and hydrolysis steps that involve physical, chemical, thermal and/or enzymatic treatment (Bothast and Saha, 1997; Galbe and Zacchi, 2002; Katzen *et al.*, 1999; Tolan, 1999; McMillan, 1994a, 1994b; Ramos and Saddler, 1994; Singh and Mishra, 1995; Stenberg *et al.*, 1998; von Sivers and

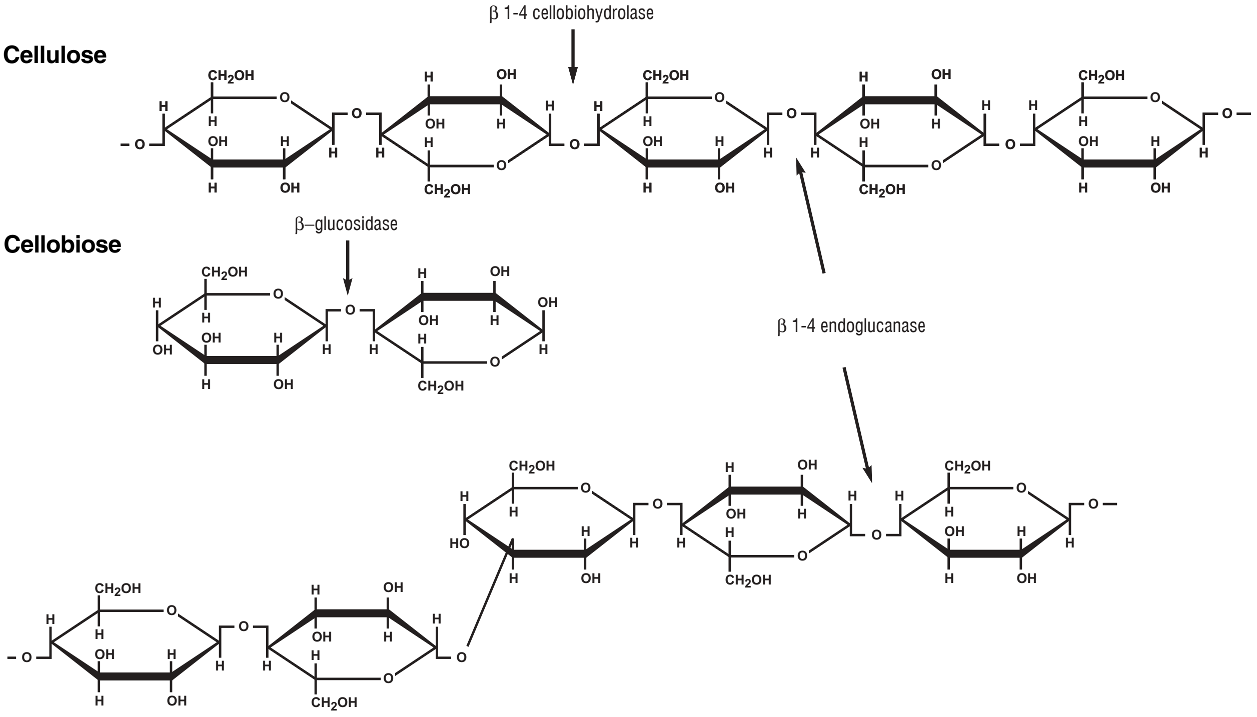

Cellulose
β 1-4 cellobiohydrolase
Cellobiose
β–glucosidase
β 1-4 endoglucanase

Figure 2. Enzymatic degradation of cellulose.

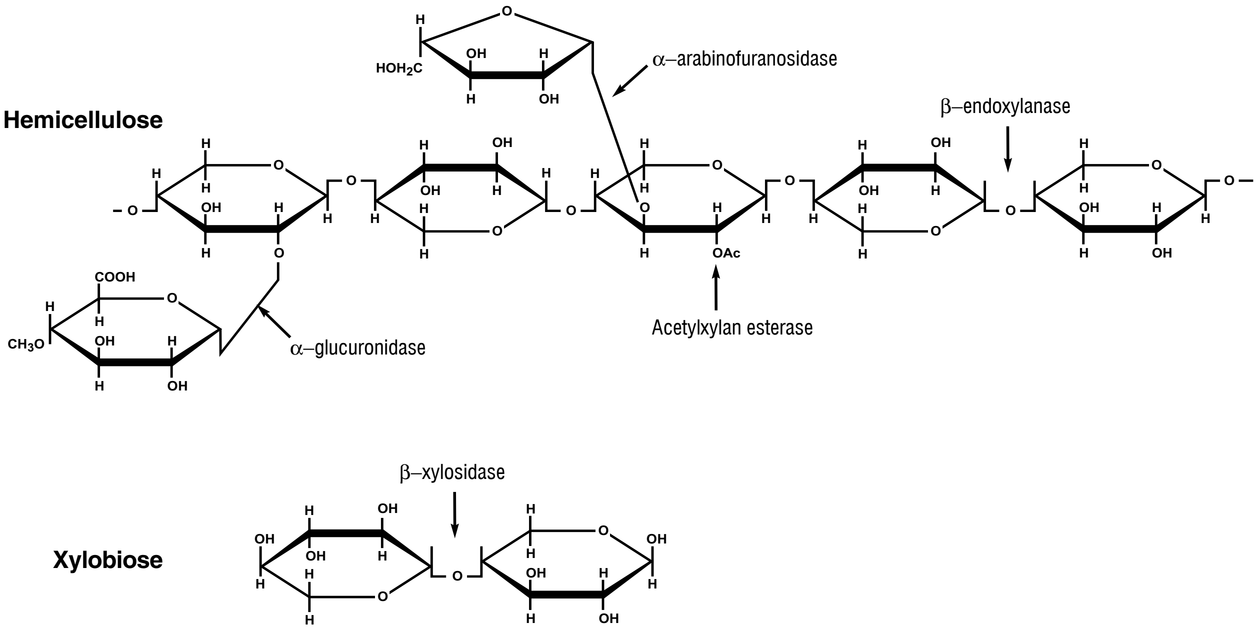

Hemicellulose
α–arabinofuranosidase
β–endoxylanase
α–glucuronidase
Acetylxylan esterase
β–xylosidase
Xylobiose

Figure 3. Enzymatic degradation of hemicellulose.

Table 3. Physiochemical methods for pretreatment of lignocellulosics.

Chemicals	Alkali	Sodium hydroxide, ammonium hydroxide
	Acids	Dilute or concentrated sulfuric acid, dilute or concentrated hydrochloric acid, nitric, phosphoric, acetic
	Oxidizing agents	Peracetic acid, sodium hypochlorite, sodium chlorite, hydrogen peroxide
	Solvents (Organosolv)	Methanol, ethanol, butanol, phenol, ethylamine, hexamethylene-diamine, ethylene glycol
	Gases	Ammonia, chlorine, nitrous oxide, ozone, sulfur dioxide.
Physical/thermal	Mechanical	Milling, grinding, extrusion, pressing
	Autohydrolysis	Steam pressure, steam explosion, hydrothermolysis, steam and mechanical sheer, pyrolysis, dry heat expansion, moist heat expansion
	Irradiation	Gamma, electron beam, photooxidation

Zacchi, 1996). A list of pretreatment methods is provided in Table 3 and the reader is urged to consult the references cited for greater detail. The enzymes involved in the hydrolysis of the polysaccharide components of lignocellulosics are covered in the next section of this chapter and some of their substrates and catalytic action sites are illustrated in Figures 2 and 3. The successful integration of the pretreatment and hydrolysis steps when combined with multistep separation and fractionation, along with simultaneous saccharification and fermentation (SSF), can result in an environmentally friendly process that yields a fermentable sugar slurry that is nontoxic or minimally toxic to ethanol producing microorganisms. The requirements for an economically viable process to meet the above criteria are as follows:

1) a well characterized lignocellulosic feedstock
2) high solids loading by employment of physical means to increase surface area
3) a treatment method to hydrolyze the carbohydrate polymers to monosaccharides while minimizing the formation of sugar degradation products (HMF, furfural, levulinic acid)
4) little or no by-product waste stream that requires waste treatment and/or disposal
5) reduced enzyme cost and usage through proper use of enzyme loading with the use of recycling and/or the use of recombinant or naturally-occurring microorganisms that produce the required hydrolytic enzymes
6) good sugar conversion and fermentation yields
7) utilization of a well designed fermentation process that takes advantage of the currently available genetically engineered micro-organisms
8) a high ethanol concentration in the final fermentation broth which would be economical to recover by conventional distillation or through the use of new technologies such as solvent extraction or membrane pervaporation.

Based on these criteria it becomes apparent that the use of lignocellulosics for the production of ethanol requires significant process integration. This is best described by the employment of a holistic approach to processing referred to as integrative bioprocessing (Abbas, 1996; Abbas, 2000). Integrative bioprocessing has numerous advantages over conventional processing in that it combines advances in chemical engineering, bioprocessing and molecular biology to develop an optimized integrated system as compared to optimizing the individual components of a process separately (Figure 4).

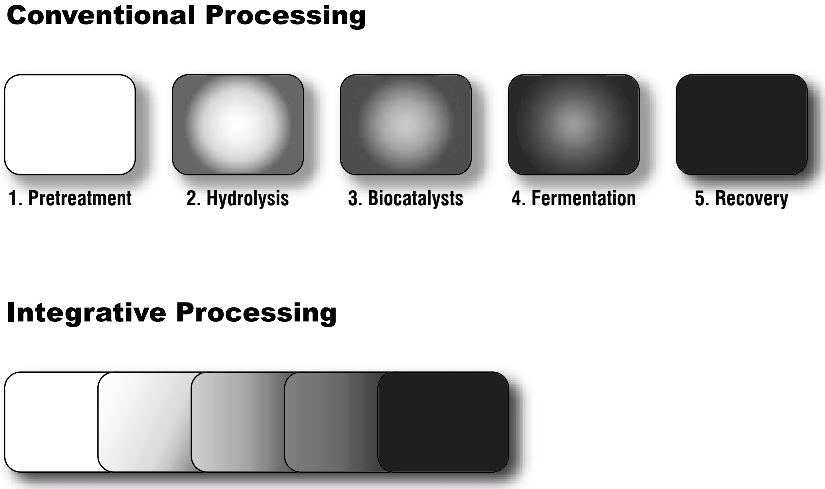


Figure 4. Illustration of integrative bioprocessing.

One of the most promising feedstocks that can meet many of the criteria listed is corn fiber hulls, a lignocellulosic by-product of the corn wet milling process (Abbas, 1996; Abbas *et al.*, 2002; Beery *et al.*, 2003; Gulati *et al.*, 1996; Moniruzzaman *et al.*, 1996; Saha *et al.*, 1998; Saha and Bothast, 1999; Werpy *et al.*, 2001). Other promising feedstocks that are currently available and meet many of the criteria listed above are agricultural residues and bioprocessing plant by-products, which include soybean hulls, wheat straw, pentosan-containing fibrous starch streams from wheat processing, corn mesa processing, fibrous by-product from dry grind tortilla flour operations, sugar beet pulp fibers, rice straw, sugar cane bagasse, spent brewers grains and dried distillers solubles from dry grind ethanol plants. Beyond the above readily available feedstocks, municipal wastes, paper and pulp wastes, corn stover and corncobs represent additional attractive feedstocks. The conversion of these feedstocks can be economically feasible provided that a cost-effective process is delineated for their collection, delivery, analysis, pretreatment, hydrolysis of the carbohydrate fractions and/or by-product disposal. The development of both cost effective hydrolytic enzymes and high solids pretreatment with good yields will be the major drivers for greater utilization of this last group of lignocellulosics.

Enzymes for the processing of lignocellulosics

The enzymatic depolymerization of lignocellulosics requires an extensive battery of microbial hydrolytic enzymes from bacterial and fungal sources that are capable of hydrolyzing cellulose, hemicellulose and lignin (Baker *et al.*, 1995; Ericksson, 1981; Galbe and Zacchi, 2002; Ghosh and Ghosh, 1992; Jeffries, 1994; Joseleau *et al.*, 1994; Lynd *et al.*, 2002; Malburg *et al.*, 1992; Nieves *et al.*, 1997; Pelaez *et al.*, 1995). These enzymes, which include glycohydrolyases, esterases and ligninases, must act on the polysaccharide backbones as well as the branched chains and substituents attached to lignin. Enzymatic digestion can be facilitated by pretreatment and hydrolysis, which enhances enzyme reaction rate by improving binding to the solubilized polymers and consequently improves process economics by reducing enzyme loading. Enzyme preparations that are known to be effective on lignocellulosics contain several exo- and endo-glycohydrolytic activities and include cellulase enzyme complexes that degrade cellulose and hemicellulases such as D-xylanases, L-arabinases, D-galactanases, D-mannases and esterases that release acetyl groups from branched sugar polymers or that release ferulic acid or/other lignin precursors (Figures 2 and 3). While an understanding of the enzymatic arsenal involved in the primary attack on lignin is still incomplete, several important enzymes are known to be involved (Ericksson, 1981; Ericksson, 1993; Hatakka, 1994; Singh and Mishra, 1995). These enzymes include the extracellular lignolytic enzymes derived from white rot fungi, which consist of lignin peroxidase (LiP), manganese peroxidase (MnP) and laccases (Ericksson, 1981; Ericksson, 1993; Fiechter, 1993; Hatakka, 1994; Odier and Artaud, 1992; Pelaez *et al.*, 1995; Youn *et al.*, 1995). Unlike the polysaccharide degrading enzymes, which are substrate specific, the enzymes that initiate degradation of lignin appear to have a broad range of substrates (Joseleau *et al.*, 1994).

Most of the current commercially available carbohydrate and lignin degrading enzymes are derived primarily from fungal sources using submerged liquid or in some cases solid state surface fermentation (Hatakka, 1994; Duarte and Costa-Ferreira, 1994; Nieves *et al.*, 1997). When tested on a lignocellulosic feedstock of interest, considerable variation has been observed in commercial enzyme preparations in the distribution of key enzyme activities and in the relative activities of cellulases and hemicellulases (Abbas, unpublished observations). For this reason careful attention must be paid to the method, substrate and conditions of the assay used by suppliers to measure enzyme activity. Whenever possible, additional information on other enzyme activities assayed should be requested. Knowledge of the microbial source and feedstock used by the manufacturer can be useful in selecting enzyme preparations for further evaluation of a targeted feedstock.

In addition to selection and use of the proper enzyme activities, care should be taken to design a process that integrates enzyme addition whenever possible into pretreatment, hydrolysis and fermentation. In view of the current limitations of commercial enzymes and their prohibitive cost, there exists the need to integrate and locate enzyme production facilities in the vicinity of, or as a part of, lignocellulosic processing plants (Tolan, 1999). The enzyme production facility should utilize a pretreated feedstock derived from the lignocellulosic of interest and the end user should employ a minimally treated or a crude enzyme preparation. This ensures a cost-effective enzyme with the necessary mixture and ratios of activities needed to process a given lignocellulosic (Adney *et al.*, 1994). Additional cost reduction in processing lignocellulosics can be realized from reduced enzyme loading by timing enzyme addition with pretreatment and hydrolysis and the use of simultaneous saccharification and fermentation (SSF). Use of SSF aids in improved enzyme catalysis by reducing feedstock inhibition from the end products, as is the case with glucose and cellobiose inhibition of cellulases.

Microbial fermentation of lignocellulosics

For several decades microbial utilization of sugars obtained from the hydrolysis of lignocellulosics for the production of fuel ethanol has been an active area of research (Corrington and Abbas, 2003; DeCarvalho *et al.*, 2002; Dennison and Abbas, 2000; Dennison and Abbas, 2003; Dien *et al.*, 1997; Green *et al.*, 2001; Ho *et al.*, 1999; Ho *et al.*, 2000a; Ingram and Doran, 1995; Ingram, 2000; Jeffries, 1985; Lai *et al.*, 1996; Wood and Ingram, 1996; Sreenath and Jeffries, 1996; Sreenath and Jeffries, 2000; Jeffries *et al.*, 1994; Jeffries *et al.*, 1995 Martin *et al.*, 2002; Palmqvist and Hahn-Hagerdal, 2000; Rogers *et al.*, 1997; Supple *et al.*, 2000; Joachimsthal and Rogers, 2000; Lawford and Rousseau, 1993a, 1993b; Lawford *et al.*, 2001; Lawford and Rousseau, 2002; Lawford and Rousseau, 2003; Yamada *et al.*, 2002; Tengborg *et al.*, 2001; Stengberg *et al.*, 2000 ; van Zyl *et al.*, 1999). This has been largely due to the absence of suitable ethanolgens that can utilize the mixture of the various pentose, hexose and higher sugars present in hydrolysates (Singh and Mishra, 1995). Research has confirmed considerable differences in the uptake and utilization of the various sugars by bacteria, yeast and molds (Ho *et al.*, 2000b; Ingram, 2000; Jeffries *et al.*, 1994; Jeffries *et al.*, 1995; Green *et al.*, 2001; Zhang *et al.*, 1998; Zhang, 2002; Picataggio *et al.*, 1994). Several strategies have been employed to remedy these limitations ranging from host selection, host modification by classical strain development approaches and genetic engineering of new strains (Ho *et al.*, 1999; Ho, 2000b; Jeffries *et al.*, 1995; Ingram, 2000; Picataggio *et al.*, 1994). While an extensive list of microbial systems that have resulted from

Table 4. Ethanolgens of interest.

Candida shehatae and *Pichia stipitis*	D-xylose fermenting yeast mutants; recombinants	T. Jeffries, University of Wisconsin
Saccharomyces cerevisiae	Recombinant D-xylose fermenting yeast	N. Ho, Purdue University; B. Hahn Hagerdal, Lund University (Sweden)
	Recombinant L-arabinose fermenting yeast	E. Boles, Heinrich-Heine University (Germany); VTT (Finland)
Zymomonas mobilis	Recombinant D-xylose/L- arabinose fermenting	NREL, US Department of Energy
E. coli/Klebsiella oxytoca	Recombinant D-xylose/L-arabinose fermenting	L.O. Ingram, University of Florida
Bacillus stearothermophilus	Recombinant D-xylose/L-arabinose fermenting	University of London, AGROL

Table 5. Fermentations of lignocellulosics.

Single or pure culture	Pentose fermenting ethanolgenic yeast or Recombinant bacteria or yeast
Co-culture	Utilize an ethanolgenic pentose fermenting yeast in combination with a brewing yeast
Isomerization followed by fermentation	Carry out isomerization at neutral to slightly alkaline pH followed by lowering pH and temperature and fermentation using brewing yeast
Simultaneous saccharification and fermentation (SSF)	Commonly employed by combining the use of free cellulases/ hemicellulases/ligninases with an ethanolgenic organism or an ethanolgen genetically engineered to utilize lignocellulosics

research in this area is outside the scope of this chapter, a short list of ethanolgens of interest is summarized in Table 4. A number of excellent references on the growth of these organisms on lignocellulosic hydrolysates and/or sugars is provided at the end of this chapter. In some instances, modifications in the fermentation process have been employed to enhance sugar uptake and utilization as well as to circumvent hydrolysate toxicity and end product toxicity. The list provided in Table 5 illustrates some of the available options that can be used in the fermentation of sugars in lignocellulosic hydrolysates by combining an appropriate hydrolysate and process.

No discussion of lignocellulosic hydrolysate fermentation is complete without addressing the major differences in the metabolism of the pentoses D-xylose and L-arabinose by fungi and bacteria. These differences are illustrated in Figures 5 and 6. In bacteria, D-xylose utilization involves the action of D-xylose isomerase followed by phosphorylation of D-xylulose by D-xylulose kinase (Amore *et al.*, 1989; Gulati *et al.*, 1996; Ho *et al.*, 1999; Singh and Mishra, 1995). By comparison the utilization of D-xylose in fungi proceeds with the action of D-xylose reductase with the formation of xylitol as the product (Bruinenberg *et al.*, 1984; Jeffries, 1985; Jeffries *et al.*, 1994; Jeffries *et al.*, 1995; Singh and Mishra, 1995; Jeppsson *et al.*, 1999). This is followed by dehydrogenation of xylitol by the action of xylitol dehydrogenase to D-xylulose, which in turn is acted on by D-xylulose kinase (Singh and Mishra, 1995; Amore *et al.*, 1989). The product of the phosphorylation, D-xylulose-5-phosphate, is assimilated into the pentose phosphate pathway, which feeds into the glycolytic pathway leading to the production of ethanol.

A significant difference in cofactor use exists between the naturally pentose-fermenting yeast such as *Pichia stipitis* and the brewing yeast, *Saccharomyces cerevisiae*, as illustrated in Figure 7. In *Pichia stipitis* and in other genera of pentose fermenting yeast, the reductases that

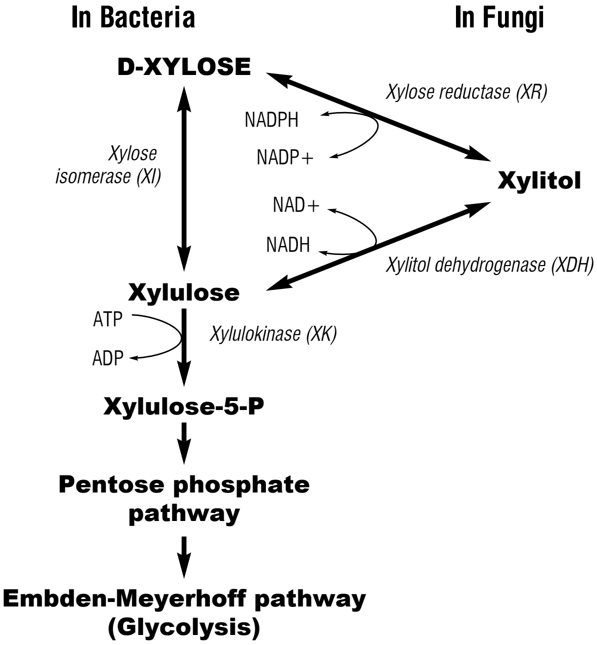

In Bacteria
In Fungi
D-XYLOSE
Xylose reductase (XR)
NADPH
NADP+
Xylose isomerase (XI)
Xylitol
NAD+
NADH
Xylitol dehydrogenase (XDH)
Xylulose
ATP
ADP
Xylulokinase (XK)
Xylulose-5-P
Pentose phosphate pathway
Embden-Meyerhoff pathway (Glycolysis)

Figure 5. Microbial D-xylose metabolism.

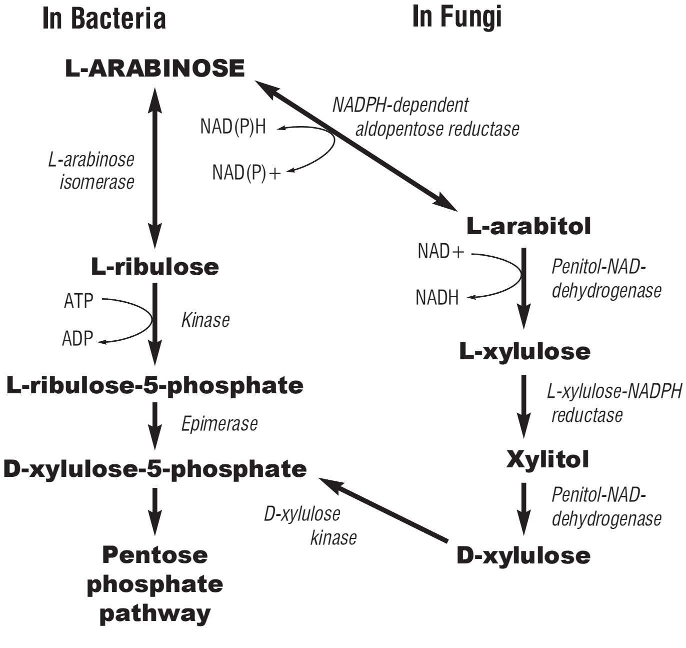

In Bacteria
In Fungi
L-ARABINOSE
NADPH-dependent aldopentose reductase
NAD(P)H
NAD(P)+
L-arabinose isomerase
L-arabitol
NAD+
NADH
Penitol-NAD-dehydrogenase
L-ribulose
ATP
ADP
Kinase
L-xylulose
L-ribulose-5-phosphate
L-xylulose-NADPH reductase
Epimerase
Xylitol
D-xylulose-5-phosphate
Penitol-NAD-dehydrogenase
D-xylulose kinase
D-xylulose
Pentose phosphate pathway

Figure 6. Microbial metabolism of L-arabinose.

reduce D-xylose to xylitol can utilize either NADH or NADPH. In contrast, in *S. cerevisiae* the host reductase that acts on D-xylose is limited to NADPH. This cofactor use in *S. cerevisiae* is responsible for the cofactor regeneration imbalance and is one of the reasons why this yeast is unable to produce significant levels of ethanol from D-xylose under anaerobic conditions (Amore *et al.*, 1989; Bruinenberg *et al.*, 1984; Jeffries, 1985). Cloning of the *Pichia stipitis* D-xylose reductase that utilizes NADH into *S. cerevisiae* improved the ability of this yeast to utilize D-xylose and resulted in improved ethanol production by the genetically engineered *Saccharomyces* strain (Table 6). Further genetic engineering of *S. cerevisiae* to increase D-xylulose kinase activity yielded new recombinant yeast strains with significant ethanol production from D-xylose (Ho *et al*., 1999; Ho *et al*., 2000a; Eliasson *et al*., 2000). Most recently, employment of metabolic flux analysis to recombinant *S. cerevisiae* grown on a mixture of D-glucose and D-xylose has been used to further delineate additional genes that can be targeted to improve ethanol production from D-xylose (Figure 8). This latest approach highlights the great potential offered by metabolic engineering to achieve further improvements in ethanol yield and productivity in recombinant Saccharomyces cerevisiae (Nielsen, 2001; Ostergaard, 2000).

Table 6. Effect of XK overexpression by *S. cerevisiae*.

+XR +XDH	Brewing yeast expressing these two genes are poor producers of ethanol.
+XR +XDH +XK	Brewing yeast expressing these three genes show greater ethanol production. Recombinants expressing a truncated XK are good D-xylose fermenters

The metabolism of L-arabinose in bacteria (outlined in Figure 6) involves the enzyme L-arabinose isomerase, which yields L-ribulose as the product (Deanda *et al.*, 1996; Saha and Bothast, 1995). Further metabolism of L-ribulose proceeds with its phosphorlyation by the action of a L-ribulose kinase to form L-ribulose-5-phosphate which is converted to D-xylulose -5-phosphate. As indicated earlier, D-xylulose-5-phosphate is assimilated further via the pentose phosphate pathway into glycolysis, which in ethanolgenic recombinant bacteria culminates in the production of ethanol. In fungi the utilization of L-arabinose involves the initial action of an aldopentose reductase with the production of L-arabitol. The metabolism of L-arabitol is carried on further through the action of a penitol dehydrogenase, an L-xylulose reductase, a second penitol dehydrogenase and finally D-xylulose kinase. The product of the last step, D-xylulose-5-phosphate, is assimilated into the pentose phosphate pathway and from there further into the glycolytic pathway.

Recently two approaches that utilized knowledge gained from L-arabinose metabolism in bacteria and fungi were successfully employed to engineer strains of *S. cerevisaie* to ferment this sugar to the ethanol. In the first approach, the bacterial genes from *Escherichia coli* (Ara B and Ara D) and *Bacillus subitilis* (Ara A) were cloned into a *S. cerevisaie* host strain (Becker and Boles, 2003). This strain was further engineered to over express Gal 2 (galactose permease) and to enhance the enzyme transaldolase activity while reducing L-

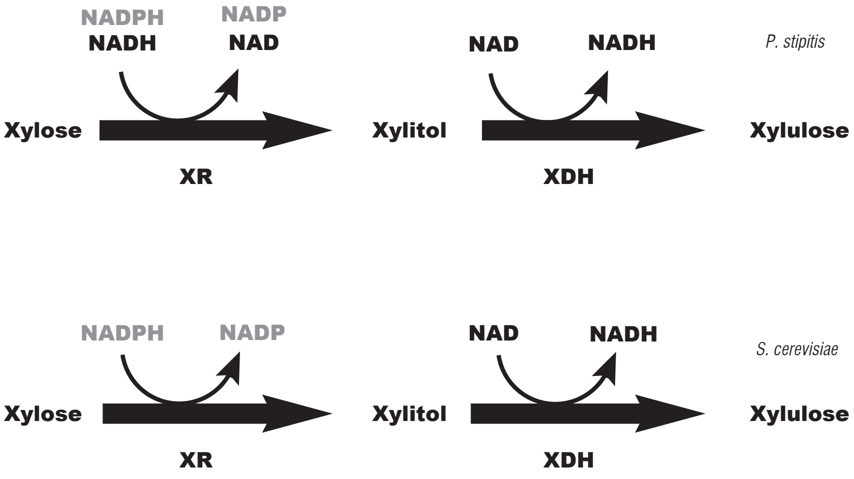


Figure 7. Xylose pathways of *Pichia stipitis* and *Saccharomyces cerevisiae.*

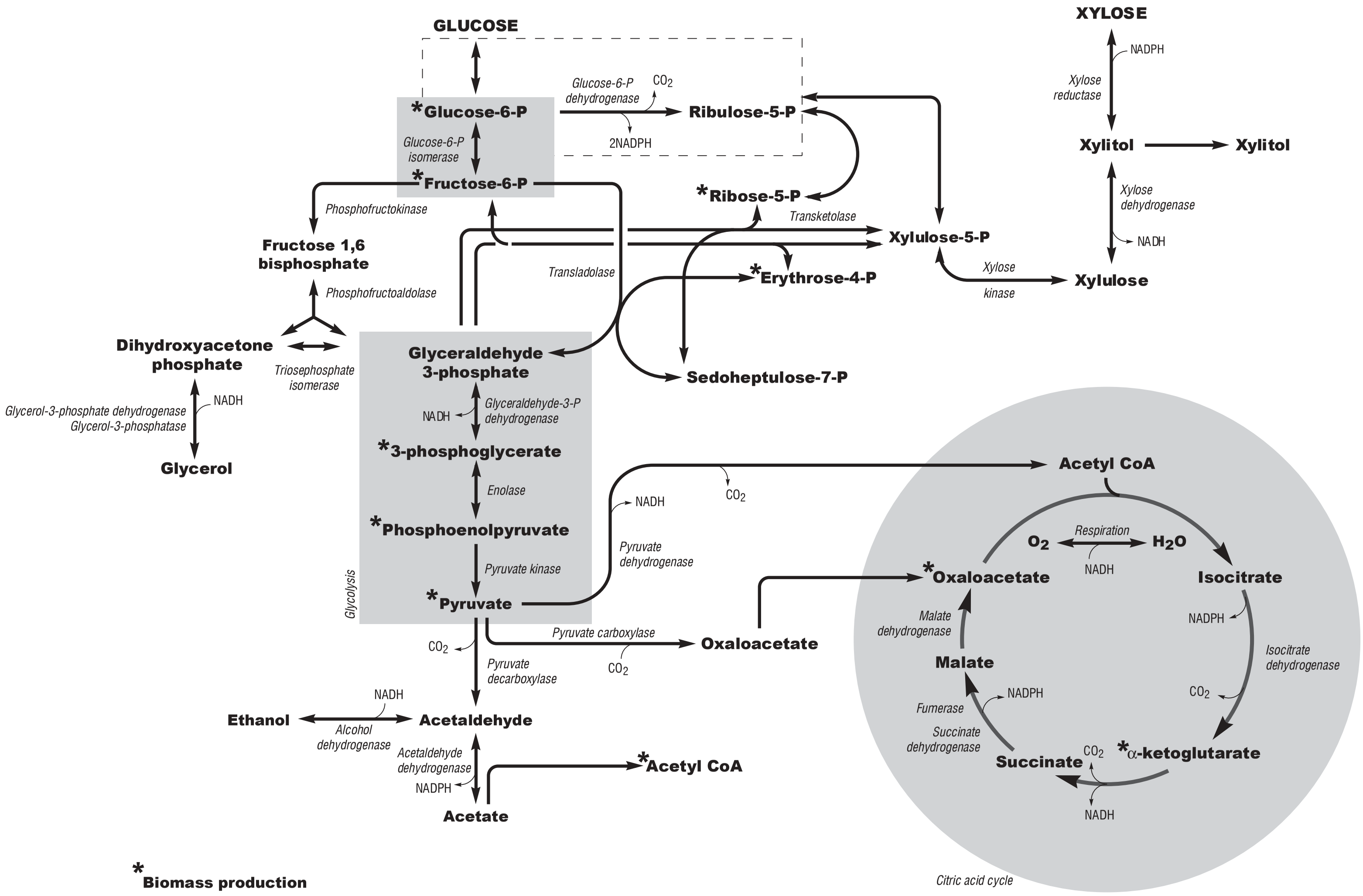

GLUCOSE
*Glucose-6-P
Glucose-6-P isomerase
*Fructose-6-P
Glucose-6-P dehydrogenase
CO_2
2NADPH
Ribulose-5-P
*Ribose-5-P
Transketolase
Xylulose-5-P
Transladolase
*Erythrose-4-P
Sedoheptulose-7-P
Phosphofructokinase
Fructose 1,6 bisphosphate
Phosphofructoaldolase
Dihydroxyacetone phosphate
Triosephosphate isomerase
Glycerol-3-phosphate dehydrogenase
Glycerol-3-phosphatase
NADH
Glycerol
Glyceraldehyde 3-phosphate
NADH
Glyceraldehyde-3-P dehydrogenase
*3-phosphoglycerate
Enolase
*Phosphoenolpyruvate
Pyruvate kinase
*Pyruvate
Glycolysis
NADH
Pyruvate dehydrogenase
CO_2
Acetyl CoA
Pyruvate carboxylase
CO_2
Oxaloacetate
CO_2
Pyruvate decarboxylase
NADH
Ethanol
Alcohol dehydrogenase
Acetaldehyde
Acetaldehyde dehydrogenase
NADPH
Acetate
*Acetyl CoA
XYLOSE
NADPH
Xylose reductase
Xylitol
Xylitol
Xylose dehydrogenase
NADH
Xylose kinase
Xylulose
*Oxaloacetate
O_2
Respiration
H_2O
NADH
Isocitrate
NADPH
Isocitrate dehydrogenase
CO_2
*α-ketoglutarate
CO_2
NADH
Succinate
Succinate dehydrogenase
Fumerase
NADPH
Malate
Malate dehydrogenase
Citric acid cycle
*Biomass production

Figure 8. Metabolic flux analysis of D-glucose and D-xylose in *Saccharomyces cerevisiae*.

ribulokinase activity. The engineered strain fermented L- arabinose to ethanol under reduced oxygen conditions with a doubling time of 7.9 hrs in a medium containing L-arabinose as the sole carbon source. In the second approach, the five genes involved in L-arabinose metabolism in fungi were cloned into a *S. cerevisiae* host strain (Richard *et al.*, 2002; Richard *et al.*, 2003). These genes were over expressed in the host strain leading to growth on L-arabinose and the production of ethanol under anaerobic conditions.

Current status of lignocellulosic research in the US

Over the past decade several initiatives that aim to improve the economics of the utilization of lignocellulosics for fuel ethanol production have been proposed and funded by the US DOE. These initiatives focused primarily on three areas:

1) sugar or feedstock development platform with most recent focus on lignocellulosics present in corn fiber hulls from corn wet mills and corn stover
2) enzyme development platform, which primarily targets improvements in cellulase enzyme technology with the goal of reducing the cost of these enzymes to bring their commercial costs in line with other industrial enzymes
3) ethanolgens development platform with a focus on yeast and bacterial recombinants with broad and improved feedstock utilization

The above DOE-funded initiatives recognize the need for further research to improve lignocellulosic feedstock pretreatment and hydrolysis as well as to further improve biocatalysts to facilitate economic scale-up for ethanol production from renewable lignocellulosics. Recently, in an attempt to realign research programs and initiatives with that of emerging biomass to fuels and chemicals industrial biorefineries, DOE has undergone an assessment of its role and is in the process of establishing new priorities. This assessment would serve to ensure that all future development in the area of lignocellulosic research would be industry driven. Over the next decade the successful integration of the research results on lignocellulosics from the DOE platforms and from DOE co-funded industry-led pilot plant biorefineries will be key to further US efforts in improving the economics of biomass conversion to ethanol and chemicals.

Concluding remarks

In the US, lignocellulosics are attractive feedstocks for further expansion of fuel ethanol production. The recent advances in pretreatment and hydrolysis when combined with the availability of new ethanolgens have increased the feasibility of hemicellulose conversion to ethanol. The conversion of cellulose continues to be economically and technically challenging due to the current high cost of commercially available enzymes as well as the energy cost for pretreatment and hydrolysis and cost of subsequent detoxification. Processing facilities that can utilize corn crop residues such as corn stover or corn wet mill biorefineries that currently produce several hundred million pounds of corn fiber hulls annually will represent the first generation of lignocellulosic-based ethanol production plants built. These facilities will be adjacent and fully integrated into existing plant operations and thereby benefit from the available infrastructure. Based on current research trends it is predicted that these plants will be in operation within the next decade and therefore will represent in the near term the successful commercialization of lignocellulosic-based biorefineries.

References

Abbas, C.A. 1996. Agribusiness by-products potential for higher value added products. 18th Symposium on Biotechnology for Fuels and Chemicals. Gatlinburg, TN (abstract).

Abbas, C.A. 2000. Integrative bioprocessing of corn fiber hulls (CFH) to ethanol. Annual Renewable Fuels Association Board of Directors Meeting. Minneapolis, MN (abstract).

Abbas, C.A., K.E. Beery, A. Rammelsberg, M. Alnajjar, J. Franz, R. Orth, A. Schmidt, T. Werpy and R. Shunk. 2002. Thermochemical hydrolysis of corn fiber. 24th Symposium on Biotechnology for Fuels and Chemicals. Gatlinburg, TN (abstract).

Adney, W.S., C.I. Ehrman, J.O. Baker, S.R. Thomas and M.E. Himmel. 1994. Cellulase assays. Method for empirical mathematical models. ACS Symposium Series 566:218-235.

Amore, R., M. Wilhelm and C.P. Hollenberg. 1989. The fermentation of xylose-an analysis of the expression of Bacillus and Actinoplanes xylose isomerase genes in yeast. Appl. Microbiol. Biotechnol. 30:351-357.

Baker, J.O., W.S. Adney, S.R. Thomas, R.A. Nieves, Y-C. Chou, T.B. Vinzant, M.P. Tucker, R.A. Laymon and M.E. Himmel. 1995. Synergism between purified bacterial and fungal cellulases. ACS Symposium Series 618:113-141.

Becker, J. and E. Boles. 2003. A modified Saccharomyces cerevisiae strain that consumes L-arabinose and produces ethanol. Appl. Env. Microbiol. 69(7):4144-4150.

Beery, K.E., C.A. Abbas, T. A. Werpy, A.J. Schmidt and R.J. Orth. 2003. Separation and conversion of corn fiber. 225th American Chemical Society National Meeting. New Orleans, LA (abstract).

Bothast, R.J. and B.C. Saha. 1997. Ethanol from agricultural biomass substrates: a review. Adv. Appl. Microbiol. 44:261-286.

Bruinenberg, P.M., P. H.M. de Bot, P. van Dijken and W. A. Scheffers. 1984. NADH-linked aldose reductase: the key to anaerobic alcoholic fermentation of xylose by yeasts. Appl. Microbiol. Biotechnol. 19:256-260.

Corrington, P. and C. Abbas. 2003. Screening of ethanolgens on corn fiber hydrolysates. 25th Symposium on Biotechnology for Fuels and Chemicals. Breckenridge, CO (abstract).

De Carvalho Lima, K.G., C.M. Takahashi and F. Alterthum. 2002. Ethanol production from corn cob hydrolysates by *Escherichia coli* KO11. J. Indust. Microbiol. and Biotech. 29:124-128.

Deanda, K., M. Zhang, C. Eddy and S. Picataggio. 1996. Development of an arabinose-fermenting *Zymomonas mobilis* strain by metabolic pathway engineering; metabolic engineering for ethanol production from agriculture residue waste. Appl. Environ. Microbiol. 62(12): 4465-4470.

Dennison, E.K. and C.A. Abbas. 2000. Evaluation of recombinant *Zymomonas mobilis* strains on corn fiber hydrolysate. 22nd Symposium on Biotechnology for Fuels and Chemicals. Gatlinburg, TN (abstract).

Dennison, E.K. and C.A. Abbas. 2003. Evaluation of recombinant microorganism ethanol fermentation of corn fiber hydrolysate. 25th Symposium on Biotechnology for Fuels and Chemicals. Breckenridge, CO (abstract).

Dien, B.S., R.B. Hespell, L.O. Ingram and R.J. Bothast. 1997. Conversion of corn milling fibrous co-products into ethanol by recombinant *Escherichia coli* strains K011 and SL40. World J. Microbiol. and Biotech. 13:619-625.

Duarte, J.C. and M. Costa-Ferreira. 1994. Aspergilli and lignocellulosics: enzymology and biotechnological applications. FEMS Microbiology Reviews 13:377-386.

Eliasson, A., C. Christensson, C.F. Wahlbom and B. Hahn-Hagerdal. 2000. Anaerobic xylose fermentation by recombinant *Saccharomyces cerevisiae* carrying XYL1, XYL2 and XKS1 in mineral medium chemostat cultures; plasmid YIpXR/XDH/XK-mediated recombinant yeast construction for improved ethanol production. Appl. Environ. Microbiol. 66(8):3381-3386.

Eriksson, K-E. 1981. Microbial degradation of cellulose and lignin. Ekman-Days 1981, Int. Symp. Wood Pulping Chem.

Eriksson, K-E.L. 1993. Lignin biodegradation and practical utilization: concluding remarks-where do we stand and where are we going? J. Biotech. 30:149-158.

Fiechter, A. 1993. Function and synthesis of enzymes involved in lignin degradation. J. Biotech. 30:49-55.

Galbe, M. and G. Zacchi. 2002. A review of the production of ethanol from softwood; alcohol production via hemicellulose, cellulose and lignin using cellulase hydrolysis process, fungus fermentation and distillation: a review. Applied Microbiol. and Biotech. 59(6):618-628.

Ghosh, B.K. and A. Ghosh. 1992. Degradation of cellulose by fungal cellulase. In: *Microbial Degradation of Natural Products* (G. Winkelmann, ed). VCH Publishers, New York, NY, U.S.A.

Green, E., N. Baghael-Yazdi and M. Javed. 2001. Fermentative production of ethanol by heterologous expression of a pyruvate decarboxylase gene in *Bacillus sp.* WO0149865A1 International patent application, 12 July, 2001

Gulati, M., K. Kohlmann, M.R. Ladisch, R. Hespell and R. Bothast. 1996. Assessment of ethanol production options for corn products. Bioresource Tech. 58:253-264.

Hatakka, A. 1994. Lignin-modifying enzymes from selected white-rot fungi: production and role in lignin degradation. FEMS Microbiology Reviews 13:125-135.

Higuchi, K. 1990. Lignin biochemistry: biosynthesis and biodegradation. Wood Sci. Technology. 24:23-63.

Ho, N.W., Z. Chen, A.P. Brainard and M. Sedlak. 1999. Successful design and development of genetically engineered Saccharomyces yeasts for effective cofermentation of glucose and xylose from cellulosic biomass to fuel ethanol. In: *Advances in Biochemical Engineering/ Biotechnology Vol. 65:163-192* (T. Scheper, ed). Springer-Verlag and Berlin Heidelberg, Germany.

Ho, N.W., Z. Chen, A.P. Brainard and M. Sedlak. 2000a. Genetically engineered Saccharomyces yeasts for conversion of cellulosic biomass to environmentally friendly transportation fuel ethanol. ACS Symposium Series, pp. 143-159.

Ho, N.W., Z. Chen and M. Sedlak. 2000b. Strategies for successful metabolic engineering of Saccharomyces yeasts to effectively co-utilize glucose and xylose from renewable cellulosic biomass for the production of ethanol and other industrial products. Am. Chem. Soc. 219th ACS National Meeting (abstract).

Ingram, L.O. 2000. Engineering bacteria for ethanol from lignocellulosics. Am. Chem. Soc. 219th ACS National Meeting (abstract).

Ingram, L.O. and J.B. Doran. 1995. Conversion of cellulosic materials to ethanol. FEMS Microbiology Reviews 16: 235-241.

Jeffries, T.W. 1985. Emerging technology for fermenting D-xylose. Trends in Biotechnology Vol.3(8):208-212.

Jeffries, T.W. 1994. Biodegradation of lignin and hemicelluloses. In: *Biochemistry of Microbial Degradation (*C. Ratledge, ed). Kluwer Academic Publishers, The Netherlands, pp. 233-277.

Jeffries, T.W., K. Dahn, W.R. Kenealy, P. Pittman, H.K. Sreenath and B.P. Davis. 1994. Genetic engineering of the xylose-fermenting yeast *Pichia stipitis* for improved ethanol production. Am. Chem.Soc. 208th ACS National Meeting (abstract).

Jeffries, T.W., B.P. Davis, H.K. Sreenath, J.Y. Cho and K. Dahn. 1995. Genetic development of the xylose fermenting yeast *Pichia stipitis*. Am. Chem. Soc. 209th ACS National Meeting (abstract).

Jeppsson, H., K. Holmgren and B. Hahn-Hagerdal. 1999. Oxygen-dependent xylitol metabolism in *Pichia stipitis*; influence of oxygen on utilization of xylitol alone, or as a mixture with xylose or glucose, for ethanol production. Appl. Microbiol. Biotechnol. 53(1):92-97.

Joachimsthal, E.L. and P.L. Rogers. 2000. Characterization of a high-productivity recombinant strain of *Zymomonas mobilis* for ethanol production from glucose/xylose mixtures. Applied Biochemistry and Biotechnology - Part A Enzyme Eng. and Biotech. 84-86:343-356.

Joseleau, J-P. , S. Gharibian, J. Comtat, A. Lefebvre and K. Ruel. 1994. Indirect involvement of ligninolytic enzyme systems in cell wall degradation. FEMS Microbiology Reviews 13:255-264.

Katzen, R., P.W. Madson and D.A. Monceaux. 1999. Lignocellulosic feedstocks for ethanol production: the ultimate renewable energy source. In: *The Alcohol Textbook*. 3rd Edition (K.A. Jacques, T.P. Lyons and D.R. Kelsall, eds). Redwood Books, Trowbridge, Wiltshire, UK, pp. 117-127.

Lai, X., L.O. Ingram and R.B. Hespell. 1996. Cloning of celliobiose utilization genes from G- and G+ bacteria: functional expression in *Escherichia coli*. Gen. Meet. Am. Soc. Microbiol. 96th Meeting (abstract).

Lawford, H.G. and J.D. Rousseau. 1993a. Production of ethanol from pulp mill hardwood and softwood spent sulfite liquors by genetically engineered *E. coli*; *Escherichia coli* application in wood pulp waste-disposal from spent sulfite liquor. Appl. Biochem. Biotechnol. 39-40:667-685.

Lawford, H.G. and J.D. Rousseau.1993b. Developments in hemicellulose bioconversion technology; ethanol production by xylose, newsprint or spent sulfite liquor fermentation by recombinant *Escherichia coli* carrying plasmid pLOI297: a review. Energy Biomass Wastes XVI: 559-97.

Lawford, H.G. and J.D. Rousseau. 2002. Performance testing of *Zymomonas mobilis* metabolically engineered for cofermentation of glucose, xylose and arabinose. Applied Biochemistry and Biotechnology - Part A Enzyme Engineering and Biotechnology 98-100:429-448.

Lawford, H.G. and J.D. Rousseau. 2003. Cellulosic fuel ethanol: Alternative process designs with wild-type and recombinant *Zymomonas mobilis*. Applied Biochemistry and Biotechnology – Part A Enzyme Engineering and Biotechnology 105-108:457-469.

Lawford, H.G., J.D. Rousseau and J.S. Tolan. 2001. Comparative ethanol productivities of different *Zymomonas* recombinants fermenting oat hull hydrolysate. Applied Biochemistry and Biotechnology- Part A Enzyme Engineering and Biotechnology 91-93:133-146.

Lynd, L.R., P.J. Weimar, W.H. van Zyl and I.S. Pretorius. 2002. Microbial cellulose utilization: fundamentals and biotechnology. Microbiol. and Molecular Biol. Rev. 66(3):506-577.

Malburg, L.M. Jr., J.M. Tamblyn Lee and C.W. Forsberg. 1992. Degradation of cellulose and hemicelluloses by rumen microorganisms. In: *Microbial Degradation of Natural Products* (G. Winkelmann, ed). VCH Publishers, New York, NY, U.S.A.

Martin, C., M. Galbe, C.F. Wahlbom, B. Hahn-Hagerdal and L.J. Jonsson. 2002. Ethanol production from enzymatic hydrolysates of sugarcane bagasse using recombinant xylose-utilizing *Saccharomyces cerevisiae*; involving sugarcane bagasse hydrolysate, detoxification, phenoloxidase laccase, recombinant xylose-utilising *Saccharomyces cerevisiae* and fermentation. Enzyme and Microbial. Tech. 31(3):274-282.

McMillan, J.D. 1994a. Conversion of hemicellulose hydrolyzates to ethanol. ACS Symposium 566:411-437.

McMillan, J.D. 1994b. Pretreatment of lignocellulosic biomass. ACS Symposium Series 566:292-324.

Monceaux, D.A and P.W. Madson. 1999. Alternative feedstocks: a case study of waste conversion to ethanol. In: The Alcohol Textbook. 3rd Edition (K.A. Jacques, T.P. Lyons and D.R. Kelsall, eds). Redwood Books, Trowbridge, Wiltshire, UK, pp.129-135.

Moniruzzaman, M., B.S. Dien, B. Ferrer, R.B. Hespell, B.E. Dale, L.O. Ingram and R.J. Bothast. 1996. Ethanol production from AFEX pretreated corn fiber by recombinant bacteria. Biotech. Letters 18(8):985-990.

Nielson, J. 2001. Metabolic engineering. Appl. Microbiol. Biotechnol. 55:263-283.

Nieves, R.A., C.I. Ehrman,W.S. Adney, R.T. Elander and M.E. Himmel. 1997. Technical communication: survey and analysis of commercial cellulase preparations suitable for biomass conversion to ethanol; cellulase complex characterization a view to its use in ethanol production from lignocellulose. World J. Microbiol. Biotechnol. 14(2):301-304.

Odier, E. and I. Artaud. 1992. Degradation of lignin. In: *Microbial Degradation of Natural Products* (G. Winkelmann, ed). VCH Publishers, New York, NY, U.S.A.

Ostergaard, S., L. Olsson and J. Nielsen. 2000. Metabolic engineering of *Saccharomyces cerevisiae*. Microbiol. Mol. Biol. Rev. 64:34-50.

Palmqvist, E. and B. Hahn-Hagerdal. 2000. Fermentation of lignocellulosic hydrolysates. II: Inhibitors and mechanisms of inhibition; the inhibitory effect of weak acid, furan derivative and phenolic compound on ethanol production by *Saccharomyces cerevisiae* from a hydrolysate substrate; a review. Bioresource Technol. 74(1):25-33.

Pelaez, F., M.J. Martinez and A.T. Martinez. 1995. Screening of 68 species of basidiomycetes for enzymes involved in lignin degradation. Mycol. Res. 99(1):37-42.

Picataggio, S.K., M. Zhang; and Mark Finkelstein.1994. Development of genetically engineered microorganisms for ethanol production. ACS Symposium Series 566:342-362.

Ramos, L.P. and J.N. Saddler. 1994. Bioconversion of wood residues: mechanisms involved in pretreating and hydrolyzing lignocellulosic materials. ACS Symposium Series 566:325-41.

Richard, P., M. Putkonen, R. Vaananen, J. Londesborough and M. Penttila. 2002. The missing link in the fungal L-arabinose catabolic pathway, identification of the L-xylulose reductase gene. Biochemistry. 2002 May 21;41(20):6432-6437.

Richard, P., R. Verho, M. Putkonen, J. Londesborough and M. Penttila. 2003. The fungal L-arabinose catabolic pathway. In: Yeast Vol 20, Number S1. XXIst International Conference on Yeast Genetics and Molecular Biology, Gothenburg, Sweden.

Richard, P., R. Verho, M. Putkonen, J. Londesborough and M. Penttila. 2003. Production of ethanol from L-arabinose by *Saccharomyces cerevisiae* containing a fungal L-arabinose pathway. FEMS Yeast Research 3:185-189.

Rogers, P.L. 1997. Bioenergy: Ethanol from biomass. Australas. Biotechnology. 7(5): 283-284.

Rogers, P.L., E.L. Joachimsthal and K.D. Haggett. 1997. Ethanol from lignocellulosics: potential for a *Zymomonas*-based process; recombinant bacterium fermentation. Australas. Biotechnol. 7(5):304-309.

Saha, B.C. and R.J. Bothast. 1995. Fuel ethanol production from L-arabinose: constraints, challenges, current status and future trends using recombinant yeast. 209th Am. Chem. Soc. Meeting (abstract).

Saha, B.C. and R. J. Bothast. 1999. Pretreatment and enzymatic saccharification of corn fiber; acid pretreatment and cellulase and beta glucosidase-mediated saccharification of maize starch to yield monomeric sugar, useful for the economic production of ethanol. Appl. Biochem. Biotechnol. 76(2):65-78.

Saha, B.C., B.S. Dien and R.J. Bothast. 1998. Fuel ethanol production from corn fiber: current status and technical prospects. Applied Biochemistry and Biotechnology - Part A Enzyme Engineering and Biotechnology. 70-72:115-125.

Singh, A. and P. Mishra. 1995. Microbial pentose utilization. Current applications in biotechnology. Vol. 33. Elsevier Science B.V., Amsterdam, The Netherlands.

Sreenath, H.K. and T.W. Jeffries. 1996. Effect of corn steep liquor on fermentation of mixed sugars by *Candida shehatae* FPL-702. Applied Biochemistry and Biotechnology Papers from 17th Symposium on Biotechnology for Fuels and Chemicals. Vol. 57/58:551-561.

Sreenath, H.K. and T.W. Jeffries. 2000. Production of ethanol from wood hydrolyzate by yeasts. Bioresource Technology 72:253-260.

Stenberg, K., C. Tenborg, M. Galbe and G. Zacchi. 1998. Optimization of steam pretreatment of SO_2-impregnated mixed softwoods for ethanol production; softwood pretreatment by SO_2 impregnation and steam. J. Chem. Technol. Biotechnol. 71(4): 299-308.

Stenberg, K., M. Bollok, K. Reczey, M. Galbe and G. Zacchi. 2000. Effect of substrate and cellulase concentration on simultaneous saccharification and fermentation of steam-pretreated softwood for ethanol production; alcohol production by *Saccharomyces cerevisiae*. Biotechnol. Bioenrg. 68(2):204-210.

Supple, S.G., E.L. Joachimsthal, N.W. Dunn and P.L. Rogers. 2000. Isolation and preliminary characterization of a *Zymomonas mobilis* mutant with an altered preference for xylose and glucose utilization; the mutant, CP4 (plasmid pZb5) M1-2, metabolizing xylose more rapidly than glucose in mixed glucose/xylose media. Biotech. Letters 22(2):157-164.

Tengborg, C., M. Galbe and G. Zacchi. 2001. Reduced inhibition of enzymatic hydrolysis of steam-pretreated softwood; for economically feasible ethanol production using *Saccharomyces cerevisiae* and enzyme. Enzyme Microb. Technol. 28(9-10):835-844.

Tolan, J.S. 1999. Alcohol production from cellulosic biomass: the Iogen process, a model system in operation. In: *The Alcohol Textbook*. 3rd Edition (K.A. Jacques, T.P. Lyons and D.R. Kelsall, eds). Nottingham University Press, Nottingham, pp. 107-116.

Van Zyl, W. H., A. Eliasson, T. Hobley and B. Hahn-Hagerdal. 1999. Xylose utilization by recombinant strains of *Saccharomyces* *cerevisiae* on different carbon sources; xylose conversion into ethanol by fermentation of recombinant *Saccharomyces* expressing *Pichia stipitis* xylose-reductase and xylitol dehydrogenase. Appl. Microbiol. Biotechnol. 52(6):829-833.

Von Sivers, M. and G. Zacchi. 1996. Ethanol from lignocellulosics: a review of the economy; alcohol production and lignocellulose degradation and saccharification. Bioresource Technol. 56(2-3):131-140.

Werpy, T.A., J. Franz, M. Alnajjar, A. Schmidt, R.J. Orth, J. Magnuson, T. P. Binder, C. A. Abbas, T. A. Stoa and B. Sedlacek. 2001. Corn fiber separation and subsequent conversion to fuels and chemicals. 23rd Symposium on Biotechnology for Fuels and Chemicals. Breckenridge, CO.

Wood, B.E. and L.O. Ingram.1996. Production of recombinant bacterial cellulases by ethanolgenic bacteria: evaluation for cellulose fermentation. Gen. Meet. Am. Soc. Microbiol. 96th Meet (abstract).

Yamada, T., M.A. Fatigati and M. Zhang. 2002. Performance of immobilized *Zymomonas mobilis* 31821 (pZB5) on actual hydrolysates produced by Arkenol technology. Applied Biochemistry and Biotechnology 98-100:899-907.

Youn, K-D, Y.C. Hah and S-O. Kang. 1995. Role of laccase in lignin degradation by white-rot fungi. FEMS Microbiol. Letters 132:183-188.

Zhang, M. 2002. Novel recombinant *Zymomonas mobilis* 31821/pZB5 strain derived from *Z. mobilis* ATCC 31821 useful for producing ethanol by fermenting xylose; xylose utilization in ethanol production by vector-mediated gene transfer, expression in host cell. WO 2002038740.

Zhang, M., C.Y. Chou, S.K. Picataggio and M. Finkelstein. 1998. New *Zymomonas mobilis* strain containing exogenous genes; for growth on xylose and arabinose for ethanol production. WO 9850524.

Chapter 6

Ethanol production from cassava

NGUYEN T. T. VINH

The Research Institute of Brewing, Hanoi, Vietnam

Introduction to cassava

Cassava (*Manihot esculenta*), which is believed to have originated in Latin America, is an important food crop in many tropical countries of Africa, South America and Asia (Dougrell, 1982). A number of attributes have made it an attractive crop for small farmers with limited resources in marginal agricultural areas:

- it is one of the most efficient carbohydrate-producing crops;
- it is tolerant of poor soil fertility and drought;
- it has the ability to recover from the damage caused by most pests and diseases;
- the roots can be left in the ground for long periods as a food reserve and thus provide insurance against famine;
- the crop is well adapted to traditional mixed cropping agricultural systems and subsistence cultivation in which farmers seek to minimize the risk of total crop failure.

Yields of cassava range from 15-150 tonnes per ha. In Brazil it can reach 80-90 tonnes; and in Colombia yields of >50 tonnes/ha are consistently obtained.

The roots are the principle edible portion of the plant and typical ranges of composition are water, 62-65%; total carbohydrate, 32-35%; crude protein, 0.7-2.6%; fat, 0.2-0.5%; fiber, 0.8-1.3%; ash, 0.3-1.3%; cellulose, 1-3.1%; polyphenol, 0.1-0.3%. Cassava chips typically contain 65-70% of starch, which makes it one of the richest sources of fermentable substrate for the production of alcohol. In nutritional terms, cassava is primarily a source of carbohydrate-derived energy, most of which is derived from starch. In addition, cassava contains a small amount of B vitamins and toxic substances. Vitamins include B_1, B_2 and B_6. The toxic compound in cassava is phazeounatine, consisting of two cyanogenic glucosides, linamarin and lotaustralin. Linamarin accounts for more than 80% of the cassava cyanogenic glucosides. Phazeounatine content ranges from 0.001-0.04 mg/100g; and is present mainly in the outer layer of bitter cultivars. Phazeounatine itself is not toxic, but it is easily degraded to release hydrocyanic acid (HCN). Typical composition of cassava chips is shown in Table 1.

Table 1. Composition of cassava chips.

Composition	%
Moisture	14
Carbohydrate	67.6
Protein	1.75
Lipid	0.87
Cellulose	3.38
Minerals	1.79

WORLD CASSAVA PRODUCTION

Total world production has increased from 70 million tonnes in 1960 to an estimated 174 million tonnes in 2001 (Table 2). Of this total, 52% is produced in Africa, 28.5% in Asia and 19.5% in Latin America. Cassava production in Asia has risen from 48.5 million tonnes in 1985 to 49.4 million tonnes in 2001 (FAO, 2001). The two major Asian cassava-growing countries, Thailand and Indonesia, have experienced the largest increases in production. Plants for producing modified cassava starch and other starch derived products were established during the period 1985 to 1990; and government support of biofuels programs in Asia will continue to prompt higher production (Sriroth, 2002).

CASSAVA UTILIZATION AS A FOODSTUFF

About 40% of cassava is used for direct human consumption, especially in processed form. Most of the remainder is destined for animal feed or processed for starch.

Global cassava utilization is estimated to have risen by about 2% in 2000, very much in line with production, given the fact that cassava food reserves are mostly kept in the ground in the form of roots until needed for harvest. Proper cassava stocks are held only in relatively modest quantities and in dried form.

Cassava global utilization as food is put at 102 million tonnes in 2000, the bulk of which is consumed in Africa in the form of fresh roots and processed products such as gari, foufou, attiéké, etc. Global cassava utilization as feed is estimated to have remained around 34 million tonnes, most of which concentrated in Latin America, the Caribbean and in the EC (Balagopalan, 2002).

Ethanol from cassava: process overview

Dried cassava chips are used in most distilleries. The cassava chips are ground to a meal - dry meal for dry milling processes and wet mash for wet mill. The fresh roots are washed, crushed into a thin pulp and then screened. In Malaysia and other countries many factories are equipped to use cassava roots.

Cassava starch/flour is gelatinized first by cooking and further converted to simpler sugars with the help of mild acids or amylase enzymes. Cassava starch, having a low gelatinization

Table 2. World cassava production (in million tonnes).

	1985	*1986*	*1987*	*1988*	*1989*	*1990*	*1999*	*2000*	*2001*
World	136.6	133.6	136.8	141.3	148.6	150.0	172.6	175.5	174.0
Africa	58.2	58.6	58.4	59.6	62.9	64.1	92.4	92.7	90.9
Ghana	3.1	2.9	2.7	2.8	3.3	3.0	7.8	7.5	7.8
Madagascar	2.1	2.4	2.2	2.2	2.3	2.3	2.5	2.2	2.4
Mozambique	3.2	3.3	3.4	3.2	3.5	3.6	5.4	4.6	4.5
Nigeria	13.5	14.7	14.0	15.0	16.5	17.6	32.7	33.9	34.0
Tanzania	6.8	6.2	6.0	6.1	6.2	5.5	7.2	5.8	5.0
Uganda	2.7	1.9	2.8	2.5	3.1	3.2	3.3	5.0	5.5
Asia	48.5	42.7	47.6	52.3	54.1	52.0	50.9	50.5	49.4
China	3.6	3.5	3.3	3.3	3.2	3.2	3.6	3.6	3.8
India	5.7	4.9	4.8	5.4	4.5	4.6	6.1	6.2	6.2
Indonesia	14.0	13.3	14.3	15.5	17.1	16.3	16.5	15.7	15.5
Phillippines	1.7	1.7	1.8	1.8	1.8	1.9	1.8	1.8	1.8
Thailand	19.3	15.2	19.5	22.3	23.5	21.9	20.3	20.2	19.2
Vietnam	2.9	2.8	2.7	2.8	2.9	3.0	1.8	2.0	2.0
Latin America	29.6	32.1	30.6	29.2	31.4	33.7	29.2	32.1	33.5
Brazil	23.1	25.6	23.5	21.7	23.4	25.4	20.9	23.4	24.6
Colombia	1.4	1.3	1.3	1.3	1.5	1.7	1.8	1.9	2.0
Paraguay	2.9	2.9	3.5	3.9	4.0	4.0	3.5	3.5	3.7

Note: Figures are approximations. Source: FAO Production Yearbooks temperature, is easily liquified and saccharified. The main advantage of cassava over any other crop for this purpose is the presence of highly fermentable sugars after saccharification. Large volumes of the saccharified starch are fed into fermentation vessels and inoculated with actively growing yeast (*Saccharomyces cerevisiae*). A continuous saccharification system is shown in Figure 1. The pH of the mash for fermentation is optimally 4–4.5 and the temperature range is 28-32°C. Alcohol is recovered from fermented mash after 48-72 hrs by distillation.

Ethanol yields are 70-110 liters of absolute alcohol per ton of cassava roots depending on the variety and method of manufacture, which compares to an expected yield of 350-400 liters/tonne from maize. The crude alcohol of cassava is described as average in quality. It has a disagreeable odor, but can be improved if the first and last fractions in the distillation process are discarded. It is utilized for industrial purposes, including cosmetics, solvents and pharmaceutical products. If production is required for human consumption, special care should be taken in handling the roots to rid them of hydrocyanic acid.

Processing cassava for alcohol production

MILLING

The purpose of milling is to break up the cassava roots to as small a particle size as possible in order to facilitate subsequent penetration of water in the cooking process. Most distilleries use hammer mills for the cassava dried chips. In contrast to corn and rice, cassava chips are soft and easy to mill. With a hammer mill, the raw material is introduced into a chamber in which a number of hammers rotate at high speed from 75-80 m/s. The collision of the hammers with the raw material causes breakdown to a meal. The mill outlet contains a retention screen that holds back larger particles until they are broken down further so that there will be a known maximum particle size in the meal. The screens are normally in the size range of 2-3 mm for a fine grind and 6-8 mm for a coarse grind. Depending on the cooking method, the target size of particles may differ. Liquefaction in response to cooking and amylase will improve with fineness of the grind, which is a significant factor in the final alcohol yield. It is possible to obtain a 5-10% difference in yield when moving from coarse to finely ground meal, as is the case with cereal grains.

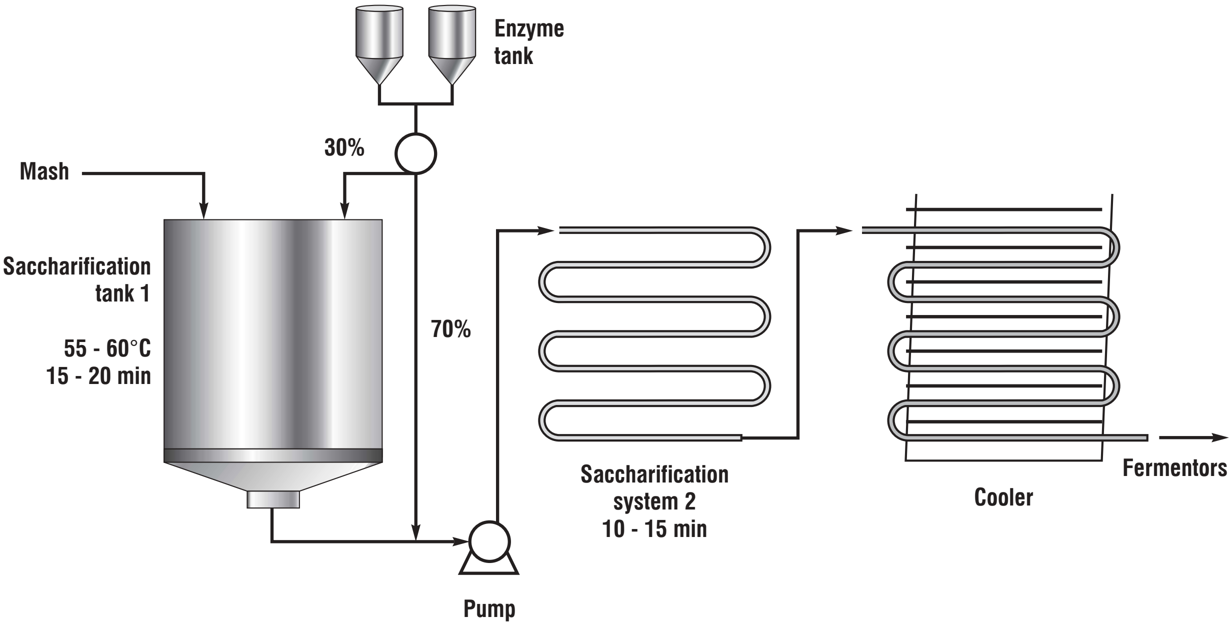


Figure 1. Typical continous saccharification system.

COOKING

Cooking is the entire process beginning with mixing the cassava meal with water through to delivery of a mash ready for fermentation. The key to cooking is to liquefy the starch so it can be pumped. There is always a protective membrane around starch granules, whether the feedstock is corn, rice, wheat or tubers such as manioc, potato, and sweet potato. Therefore the essential purpose of cooking is to break the membrane, releasing the starch.

Gelatinization is the phenomena by which a granule of starch swells in presence of water and heat so that surface area exposed to enzymes is increased. During gelatinization water penetrates the granule making the breakdown of the starch easier for the α-amylase. Starch is composed of amylose and amylopectin. While starch in corn contains 85-90% amylopectin, cassava starch contains 70% amylopectin. For each type of raw material there is a typical gelatinization temperature range, which depends on starch granule size. When granule size is small gelatinization temperatures are lower. Gelatinization temperature range of corn starch is 67-80°C, rice starch 65-85°C, and 61-70°C for cassava starch. In addition, gelatinization temperature depends on the electrolytes in solution. Alkaline substances decrease gelatinization temperature, while sugars increase gelatinization temperature.

When cooking in weak acid conditions, cellulose normally cannot be hydrolyzed. Hemicellulose is hydrolyzed by xylanase in the presence of H^+ and high temperatures forming dextrins and lower molecular weight substances including arabinose and xylose.

During cooking, a small amount of starch will be converted to sugars by endogenous amylase. Sucrose, glucose, fructose and a little maltose are formed in cooking. At high temperatures these sugars may be converted into caramel, furfural, oxymethyl furfural and other compounds. The rate of conversion depends on temperature and pH. Experience has shown that adjusting mash pH to 3.5 may reduce the rate of sugar conversion (5% for glucose and 26% for fructose). If pH is raised to 6.5, then conversion rate will be 80% for glucose and 90% for fructose.

Cooking procedures for cassava

There are three methods for cooking cassava: batch, semi-continuous and continuous. The ratio of water to material is normally 3.5:1-3:1 with amylase. The batch method is still used in many countries, but has the disadvantages of long cooking times at high temperature with accompanying sugar loss. The continuous method requires strict control. The heat-stable amylase is popular for alcohol plants, and is used at a rate of 0.02–0.03%. Around 80% of the amylase is used for the first liquefaction and 20% is added for additional liquefaction after boiling and cooling to 90°C.

Liquefaction and saccharification

Hydrolysis by the endoenzyme α-amylase is random and rapid, reducing starch to detrins of varying chain length. The shorter the chain length, the less work remains for the exoenzyme glucoamylase, which releases single glucose molecules by hydrolyzing successives α-1,4 linkages beginning at the non-reducing end of the dextrin chain. Glucoamylase also hydrolyzes α-1,6 branch linkages, but at a much slower rate.

Recently simultaneous saccharification and fermentation (SSF) of cassava has been of interest as a means of reducing the cost and time involved in ethanol production. In an experiment by Keawsompong and co-workers (2003) in Thailand, conventional liquefaction/saccharification was compared with an SSF process that employed Rhizozyme^{TM}, a suface culture glucoamylase targeted for the temperature and pH conditions of the fermentor. After 48 hrs of fermentation, similar amounts and conversion efficiencies were obtained, however by leaving out the saccharification step, the SSF process was completed 25% faster than the conventional method of sequential liquefaction and saccharification (Figure 2). Yield increases were also observed, presumably because other materials became available for fermentation. At 36 hrs the conventional system had 8-9% ethanol compared to 10%+ in the Rhizozyme^{TM} fermentor. Cell counts were also significantly higher.

FERMENTATION

After saccharification, mash is cooled down to

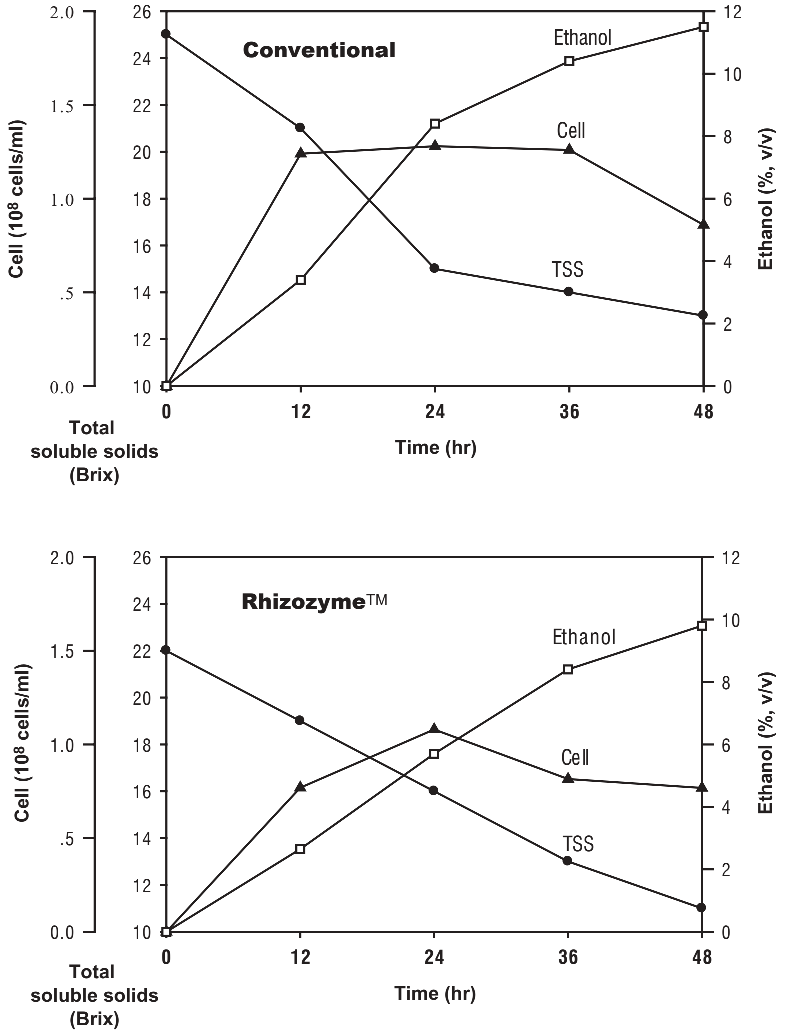


Figure 2. Changes in total soluble solids (TSS), brix, cell count and ethanol during cassava fermentation by yeast in a conventional (top) liquefaction and saccharification process and a simultaneous saccharification and fermentation (bottom) process using Rhizozyme™. The SSF process required 25% less time (from Keawsompong *et al.,* 2003).

28-32°C and is pumped into the fermentor. Fermentation is the critical step in a distillery. It is here that yeast converts sugar to ethanol, carbon dioxide and other by-products.

Saccharomyces cerevisiae is typically used for ethanol fermentation from cassava. During fermentation, foaming can be a problem. The fermentation media from cassava is usually low in nitrogen, so yeast food or other nitrogen sources should be used. Fermentation time is normally of 56-72 hrs.

As with other fermentations, infections are of concern. Lactoside™, a broad-spectrum antimicrobial, is added at 2-3 ppm in fermentors in some distilleries.

DISTILLATION

Distillation procedures are those standard throughout the industry. Cassava poses no special problems in the still. Yield is around 94-95% of calculated values.

References

Akpan, I., M.J. Ikenebomeh, N. Uraih, and C.O. Obuekwe. 1988. Production of ethanol from cassava whey. Acta Biotechnol. 8(1):39-45.

Balagopalan, C. 2002. *Cassava Utilization in Food, Feed and Industry*. CCAB International.

Dougrell, L.M. 1982. Cassava - modern agricultural system development towards ethanol production in Australia. International Alcohol Fuel Technology Symp. NZL, p.63-72.

FAO/GIEWS - Food Outlook No 3, June 2001, p.9.

Keawsompong, S., K. Piyachomkwan, S. Walapatit, C. Rodjanaridpiched and K. Sriroth. 2003. Ethanol production from cassava chips: simultaneous saccharification and fermentation process. BioThailand Conference, Pattaya, Thailand.

Sriroth, K., K. Piyachomkwan, K. Sangseethong and C. Oates. 2002. Modification of cassava starch. X International Starch Convention, 11-14 June 2002, Kracow, Poland.

Chapter 7

Whey alcohol - a viable outlet for whey?

JACK O'SHEA

Alltech Inc., Dunboyne, County Meath, Ireland

Introduction

Whey is the main by-product from casein and cheese manufacture. In the past, cheese making was a traditional cottage industry using the milk from various animals such as goats, camels and reindeer. Today cheese is usually made in large production facilities from cow's milk by the application of microbiology and chemistry. Casein, the chief milk protein, is coagulated by the enzymatic action of rennet or pepsin, by lactic acid producing bacteria, or by a combination of the two. The next step involves separating the curds from the whey, a process facilitated by heating, cutting, and pressing. With the separation of the curds most of the butterfat and a number of the other constituents are removed. The butterfat usually comprises only 3-4% by volume of whole milk. Whey makes up to 80-90% of the total volume of milk that enters the cheese making process and contains about 50% of the nutrients. These nutrients are soluble whey protein, lactose, vitamins and minerals. Whey from cheese and rennet casein is known as sweet whey; and the manufacture of acid casein yields acid whey. Acid whey is produced by removal of the fat by centrifugation to make butter. The milk is then acidified to coagulate the protein. This is then centrifuged off as casein, leaving acid whey.

Table 1. Approximate composition of whey.*

Component	*%*
Water	94.50
Dry matter	5.75
Protein	0.80
Lactose	4.40
Ash	0.50
Fat	0.05

*This is an average analysis, the type of cheese produced will influence composition of the whey.

Approximately 100 kg of milk are required to produce 10 kg of cheese. Quantities of milk produced in the top 40 milk producing countries in the world are outlined in Table 2. The top 40 cheese producing countries are listed in Table 3. The whey production from all this milk is considerable and can present a disposal problem. The challenge to dairy production plants is to dispose of whey in an environmentally friendly and economic manner. The problems facing whey producers and the uses for whey are explored below.

Problems with whey

Whey produced by dairies has historically been and remains today a potential pollution problem.

Table 2. International production of milk (thousand tonnes).[1]

Country		*1995*	*1996*	*1997*
1	USA	70500	70003	71072
2	India	32000	35500	34500
3	Russian Federation	39098	35590	34000
4	Germany	28621	28799	28700
5	France	25413	25083	24957
6	Brazil	16985	18300	19100
7	Ukraine	17060	15592	16300
8	United Kingdom	14683	14680	14849
9	Poland	11642	11696	12099
10	New Zealand	9285	9999	11131
11	Netherlands	11294	11013	10922
12	Italy	10497	10799	10531
13	Argentina	8792	8947	9795
14	Turkey	9275	9466	9466
15	Australia	8460	8986	9303
16	Japan	8382	8657	8642
17	Mexico	7628	7822	8091
18	Canada	7920	7890	7800
19	China	6082	6610	6946
20	Spain	6150	6084	5997
21	Columbia	5078	5000	5408
22	Irish Republic	5415	5420	5380
23	Romania	4646	4676	5126
24	Belarus	5070	4908	4850
25	Denmark	4676	4695	4632
26	Pakistan	4223	4379	4540
27	Switzerland	3913	3862	3913
28	Iran	3450	3809	3897
29	Kazakhstan	4576	3584	3584
30	Belgium/Luxembourg	3644	3682	3477
31	Sweden	3304	3316	3334
32	Uzbekistan	3665	3183	3200
33	Czech Republic	3155	3164	3164
34	Austria	3148	3034	3090
35	Sudan	2760	2880	2880
36	South Africa	2794	2592	2720
37	Finland	2468	2431	2463
38	Kenya	2170	2210	2210
39	Korea	1998	2034	2072
40	Chile	1890	1924	2040
Total world milk production		**421,810**	**422,299**	**426,181**

[1] National statistics, Eurostat, 1999.

Table 3. International cheese production (thousand tonnes).[1]

Country		*1995*	*1996*	*1997*
1	USA	3493	3627	3627
2	France	1617	1635	1660
3	Germany	1453	1531	1591
4	Italy	982	985	940
5	Netherlands	700	709	713
6	Russian Federation	477	477	477
7	Argentina	369	376	405
8	Poland	354	397	397
9	United Kingdom	362	377	378
10	Egypt	344	346	349
11	Canada	301	312	323
12	Denmark	311	299	291
13	New Zealand	197	239	274
14	Australia	234	261	270
15	Spain	240	247	246
16	China	202	202	206
17	Iran	196	196	197
18	Greece	202	192	192
19	Czech Republic	137	139	139
20	Turkey	136	139	139
21	Switzerland	132	133	133
22	Mexico	126	123	123
23	Sweden	129	127	118
24	Japan	105	109	114
25	Austria	101	100	107
26	Irish Republic	84	98	97
27	Israel	92	92	92
28	Norway	85	84	89
29	Finland	96	95	88
30	Hungary	83	88	88
31	Syria	66	75	86
32	Sudan	75	76	76
33	Belgium/Luxembourg	72	73	76
34	Bulgaria	82	71	72
35	Ukraine	88	71	71
36	Venezuela	103	70	70
37	Portugal	67	67	65
38	Brazil	60	60	60
39	Columbia	51	51	51
40	Chile	48	49	51
Total world		**14,052**	**14,398**	**14,541**

[1] National statistics, Eurostat, 1999.

As a consequence, the majority of cheese producing countries have strict laws defining the permitted biological and chemical oxygen demand (BOD and COD) limits for the effluent from dairy plants. A cheese manufacturer's ability to dispose of the whey produced may be the limiting step in the volume of cheese that can be produced.

A number of problems are common to all whey users. The first complication is that the whey is extremely perishable. This is especially true for cheese whey, which contains bacteria added during cheese production. The whey must therefore be protected from biological degradation; and the most effective method of protection is pasteurization. Alternatively, the whey can be cooled to 4°C or protected by the use of chemical additives, e.g., hydrogen peroxide. A second problem with whey is the very high water content, over 93%. This means that transport and concentration costs are high, and form a large proportion of the total processing cost. Another factor influencing the economics of whey processing in many countries is one of seasonality. Most cheese production occurs between March and September. This means that the processing plant is used for only half of the year. Finally, cheese whey contains casein fines and fat that must be removed prior to most processing methods. This is not particularly a disadvantage, since the recovered fines can be added back to the cheese or used to make processed cheese while the separated fat can be used to make whey butter (Figure 1). The fines are usually separated on vibrating screens and the fat by centrifugation.

Uses for whey

WHEY AS AN ANIMAL FOOD

One solution to the whey problem is to sell it to pig farmers, however there are many limitations to the use of whey as a pig feed. Due to its low dry matter content, whey-fed pigs typically produce very large volumes of urine. Many piggeries are not in a position to deal with the

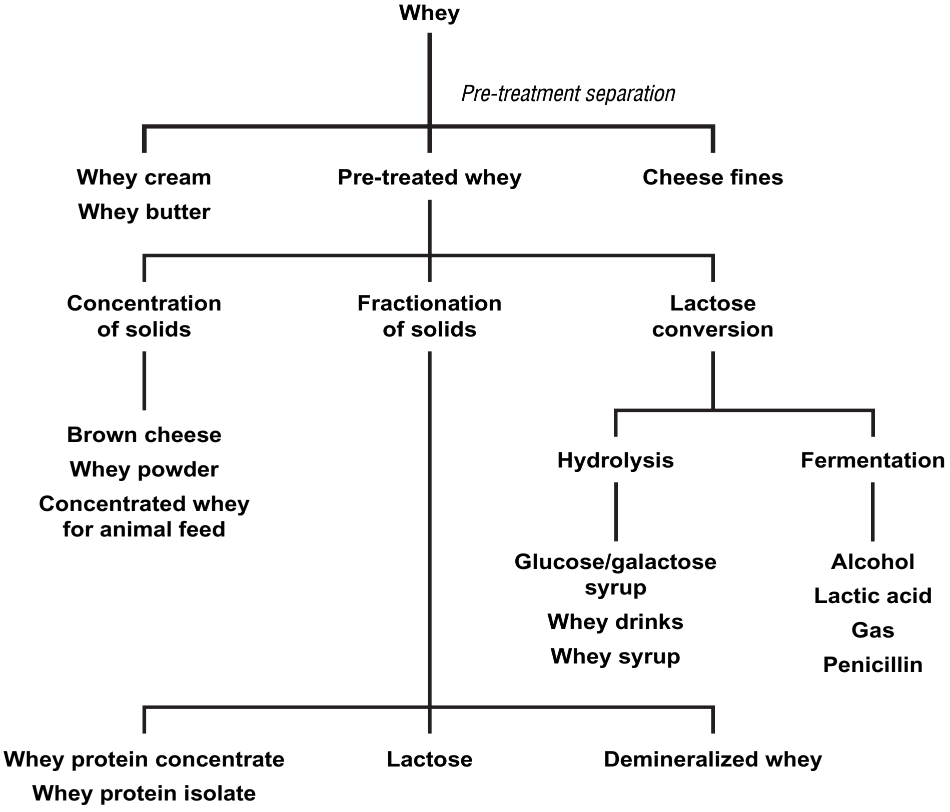

Whey
Pre-treatment separation
Whey cream
Whey butter
Pre-treated whey
Cheese fines
Concentration of solids
Fractionation of solids
Lactose conversion
Brown cheese
Whey powder
Concentrated whey for animal feed
Hydrolysis
Fermentation
Glucose/galactose syrup
Whey drinks
Whey syrup
Alcohol
Lactic acid
Gas
Penicillin
Whey protein concentrate
Whey protein isolate
Lactose
Demineralized whey

Figure 1. Uses for processed whey (adapted from Nielson, 1997).

high humidity in barns housing whey-fed pigs, and respiratory problems are a very real deterrent to maximizing profits from intensive piggeries. In addition, the whey supply in many regions is seasonal; it dries up between October and April. The pig farmer is therefore vulnerable to these fluctuations in feed supply, and is more likely to opt for feed sources available all year such as barley plus soya. At the same time, the summer surplus of whey cannot be utilized by the pig farmer with a fixed number of pig spaces. In addition, whey is deficient in protein and fat-soluble vitamins and requires balancing with appropriate additives. The difficulties recently experienced by the European pig industry indicate how volatile this industry is and that it is not a guaranteed 'sink' for whey. Many whey producers would prefer to have several relatively secure outlets for their whey.

WHEY POWDER

Whey is processed by several methods. One of the most common methods is the production of whey powder by drying. Table 4 outlines whey powder production in Europe from 1995 to 1998. Drying sounds like a simple process, but a number of stages are necessary to produce a high quality powder. The free-flowing whey powder resulting from drying has a number of applications in the food industry, including incorporation into dry mixes, baked foods, confections, ice cream and processed cheese. Whey may also be made into cheese such as the Scandinavian primost and mysost (Edelman and Grodnick, 1986).

Reverse osmosis may also be used to concentrate whey. It works on the same principle as ultrafiltration except that membranes are such that only water is allowed to pass through them. Reverse osmosis is cheaper than thermal evaporation for removing up to 70% of the water from whey. Savings are therefore possible by using reverse osmosis for pre-concentration before evaporation or membrane processing (Burgess, 1980).

LACTOSE MANUFACTURE

One of the oldest methods of whey utilization is the manufacture of lactose. The first stage is concentration of the whey by low temperature evaporation, which ensures that the whey protein is not damaged. The concentrate, containing 60-65% solids, is fed to a crystallization tank and seeded with small crystals of lactose. Edible

Table 4. Whey powder production in Europe (thousand tonnes).[1]

Europe (15 countries)	*1995*	*1996*	*1997*	*1998 (estimated)*
Production	1280	1310	1280	1310
Consumption	1235	1235	1172	
Import	539	534	485	
of which, from outside EU	9	2	2	
Export	654	703	703	
of which, from outside EU	54	77	110	100

[1] Eurostat, 1999.

Table 5. Production of lactose in Europe (1995-1998) (thousand tonnes).[1]

Europe (15 countries)	*1995*	*1996*	*1997*	*1998 (estimated)*
Production	280	310	330	330
Consumption	195	225	230	240
Export to outside EU	85	85	100	90

[1] Eurostat, 1999.

lactose is able to absorb flavor; and it is therefore used in certain applications in the food industry. Lactose may also be used as a wine sweetener. For pharmaceutical applications, e.g., tablet formulation, the lactose crystals must be further refined to remove traces of protein and minerals. Table 5 outlines lactose manufacture in Europe from 1995 to 1998.

LACTOSE HYDROLYSIS

Lactose is not very sweet compared to cane sugar or even its constituent monosaccharides glucose and galactose. The sweetness of lactose can be considerably increased by hydrolysis. A number of technologies are available for lactose hydrolysis. These can be classified as either enzymatic methods using ß-galactosidase or ion exchange methods (Burgess, 1980).

DEMINERALISED WHEY

One of the main disadvantages of whey as a food product is its high mineral content. Whey demineralisation has opened up the baby food market to whey producers. Two different technologies may be used for the demineralisation of whey, electrodialysis and ion exchange. Of these techniques most manufacturers use electrodialysis. In the ion exchange process whey is passed through a column containing a strong cation resin and then through a column containing a weak anion resin. The former absorbs the cations in the whey and releases hydrogen ions, while the latter neutralizes the hydrogen ions and absorbs the anions. The advantage of ion exchange lies in its relative simplicity and low capital cost. However, the regeneration costs are high and a large volume of waste is generated. Ion exchange is probably cheaper than electrodialysis for high levels of demineralization. In contrast, the capital costs of electrodialysis are high while running costs are relatively low.

PROTEIN RECOVERY FROM WHEY

Whey protein is a relatively high value product. Ultrafiltration is one of the processes used to extract whey proteins. Whey proteins with a molecular weight of 12,000-13,000 are extracted, leaving the permeate suitable for fermentation. The main feature of ultrafiltration is the semi-permeable membrane, usually made of cellulose acetate, polyamide or other copolymers. The membrane acts as a filter that is permeable to the low molecular weight salts and lactose, but impermeable to the higher molecular weight proteins. Pressure is applied to the whey side of the membrane and water, salts and lactose are forced through it while protein is retained. The protein concentration in the whey therefore increases in proportion to its reduction in volume and a whey protein concentrate containing up to 60% protein on a dry weight basis can be produced. After ultrafiltration, the whey protein concentrate is evaporated to 40-50% total solids by low temperature vacuum evaporation and spray-dried. The whey proteins have a high nutritional value.

ALCOHOL PRODUCTION

After ultrafiltration there remains a large volume of permeate that needs to be processed. The protein-free permeate is a yellow liquid containing 90% of the whey solids, including most of the lactose. The composition of whey permeate (Table 6) and that of skim milk are essentially similar.

Table 6. Average composition of whey permeate.

Component	*%*
Lactose	4.8
Protein	0.6
Non-protein nitrogen	0.1
Ash	0.5
Fat	0.5
Lactic acid	0.15
Total solids	5.7

It has been known for some time that whey can be fermented to produce alcohol; but in the past synthetic alcohol produced from crude oil has been cheap. Following the energy crisis in the 1970s, the price of synthetic alcohol increased, making fermentation alcohol production processes more economically attractive. Whey previously regarded as a waste product came to be viewed as a potential substrate for alcohol production. The technology was readily available; and today a number of industrial plants successfully produce alcohol from whey permeate.

Lactose is the major constituent of whey permeate and accounts for approximately 70% of the total solids. It must be stressed that this is an average analysis for sweet whey. The type of cheese produced will obviously influence the whey composition. Small amounts of other nutritionally desirable materials including riboflavin, amino acids and relatively high levels of calcium and phosphorus are also present in whey. To obtain the maximum reduction in BOD, it is necessary to develop a process whereby practically all the lactose is converted to alcohol.

In practice, a lactose content of 4.3-5.0% is obtained in the permeate. Using the yeast strain *Kluyveromyces marxianus* a yield of 86% is attainable from an initial lactose concentration of 4.3%. This yeast has the ability to ferment lactose, a disaccharide composed of glucose and galactose (Figure 2).

The alcohol concentration following fermentation of permeate is low thus requiring a very high energy input in the subsequent distillation. On the other hand, the pollution problem is greatly decreased with the total solids (lactose and protein) being reduced to 10% of that initially present.

Biomass produced during the fermentation will add to the effluent load. Whey pH ranges from 4.6 to 5.6. The temperature range for fermentation can be 25-35°C. Using a batch process the fermentation time will be between 18 and 24 hrs. At the end of fermentation the yeast cells may be separated for re-use. If a yield of 80% is attained from a lactose concentration of 4.4%, then production of 1 liter of 100% ethanol requires 42 liters of whey. The quality of the alcohol produced is very good with only very low levels of acetaldehyde and fusel oils. The permeate can be further concentrated; and the higher lactose content will increase alcohol content and thus lower distillation costs. This is very useful where permeate must be transported to alcohol plants.

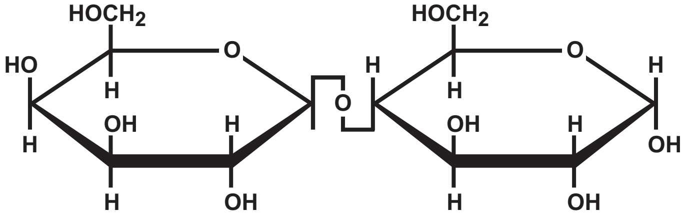
One of the greatest difficulties facing a whey alcohol plant manager is infection. In certain instances there will be a carryover of lactic acid bacteria from the cheese plant. These microorganisms grow very easily on whey permeate thus reducing the amount of substrate available for alcohol production. Infection in such fermentations is usually controlled by using antibiotics.

Not withstanding such difficulties, utilization of whey as a substrate for alcohol production on a worldwide basis has become increasingly popular. This is evidenced by the diverse geographical locations of whey alcohol plants, examples of which are given in Table 7.

Considerations relevant to construction of a whey alcohol plant

EFFLUENT TREATMENT

Although ultrafiltration removes the protein from



α-Lactose

$$C_{12}H_{22}O_{11} + H_2O \rightarrow 4C_2H_5OH + 4CO_2$$

Lactose + water   Alcohol + carbon dioxide

Figure 2. Structure of lactose and its conversion to alcohol by *Kluyveromyces marxianus.*

Table 7. Alcohol plants using whey as a substrate.

Ireland	Carbery Milk products, of Ballineen, County Cork, operate an alcohol plant using cheese whey as substrate. This alcohol is used for vodka and cream liqueurs (Murtagh, 1995).
United States	A plant to produce industrial alcohol from whey has been installed in California in one of the largest cheddar cheese plants in the world. This plant processes 1,000,000 kg of milk per day into cheese. The whey from the process is converted into permeate and protein by ultrafiltration. The protein is dried and the lactose in the permeate is converted into alcohol. The alcohol is then distilled in a distillation plant capable of processing 200,000 liters of whey per day, producing 1.5 million liters of pure alcohol per year.
New Zealand	Whey is the main by-product of the New Zealand dairy industry and is produced in the manufacture of cheese and casein. Anchor Ethanol was the first company in the southern hemisphere to manufacture alcohol from casein whey on a commercial basis. Anchor Ethanol began production at Anchor Products Repora in 1980 and a second plant was commissioned at Anchor Products Tirau the following year. These plants produce a combined total of 10 to 11 million liters of ethanol per year, over half of which is exported. This alcohol is the basis for a range of alcohol beverages. It is also used in the manufacture of solvents, methylated spirits, white vinegar, surgical spirit, food colouring, deodorants, perfumes, aerosols and pharmaceutical products (New Zealand Dairy,1999).
Russia	In Russia the range of feedstocks used for alcohol production is very diverse, including grains, fruit, whey, wine and molasses (Berg, 1999).

whey and the fermentation process converts the main ingredient (lactose) into alcohol, biomass is produced. The other ingredients of whey will contribute to the BOD/COD of the final effluent from the alcohol plant; and adequate effluent treatment facilities must be provided. One option is to use an anaerobic digestion system. This will convert a portion of the BOD into gas that can be used to fuel the boilers in the distillation plant.

MARKETS FOR ALCOHOL

The alcohol markets in the US and Europe are very different. Because of government intervention in the US, ethanol production has grown from an insignificant amount in 1978 to a record 6.4 billion liters in 1998. In Europe the situation is very different. There has been no such government intervention and an oversupply of alcohol has existed for some time. Until recently a large portion of this alcohol was sold to Russia, but this market has diminished. The low price of oil has helped the majority of alcohol producers to keep production costs down; however as oil prices rise these production costs will also increase.

In the US, ethanol was promoted as a solution for a variety of complex problems. Among them: US dependence on foreign oil supplies, which was made apparent by two oil crises of the 1970s, and the low gasoline octane ratings caused by reduced use of lead after the approval of the Clean Air Act in 1977. Ethanol production from corn was seen as a way to boost farm incomes caused by the grain surplus in the wake of the Soviet embargo. Also, addition of ethanol to gasoline was seen as a means to reduce air pollution. These provide a ready market for any alcohol produced. Within the EU there has been much discussion about bio-ethanol, but only recently has there been any commitment to bio-fuels. Tax concessions for pilot plants producing bio-fuels have been allowed; and as a consequence new bio-fuel projects have been announced in the Netherlands, Sweden and Spain. France has made the most progress. In 1996 the French approved a draft law that made the use of oxygenated components in fuel mandatory by 2000.

Conclusions

The attachment of a whey alcohol plant to a dairy plant producing the appropriate substrate solves a number of major problems confronting whey producers. Alcohol production provides a guaranteed outlet for the whey that is not subject to the fortunes of pig markets or other elements outside their control. Whey production (or disposal) does not form a limiting step in cheese or casein production. The whey protein recovered by ultrafiltration is a valuable item, the sale of which contributes to process economics. There is also a universal move towards bio-fuels, therefore the markets for alcohol should be increasingly secure in the future.

References

Berg, C. 1999. World Ethanol Production and Trade to 2000 and Beyond. *http://www.distill.com/berg.*

Edelman, E. and S. Grodnick. 1986. The Ideal Cheese Book. *http://cbs.infoplease.com/ce5/CE010398.html.*

Eurostat. 1999. Institut National de Statistique, Brussells, Belgium, *http://europe.eu.int/en/comm/eurostat/eurostat.html.*

Burgess, K.J. 1980. Uses of waste dairy products. Technology Ireland, June, pp. 43-44.

Murtagh, J.E. 1995. Molasses as a feedstock for alcohol production. In: *The Alcohol Textbook* (T.P. Lyons, D.R. Kelsall and J.E. Murtagh, eds). Nottingham University Press, Nottingham, UK. pp. 27-34.

New Zealand Dairy.1999. *http://www.nzdairy.co.nz/public/educational/whey/ethanol/Ethanol~t.htm.*

Nielson, W.K. 1997. Whey processing. Technical Bulletin. APV Ireland Ltd, Dublin.

Chapter 8

Treatment and fermentation of molasses when making rum-type spirits

ROBERT PIGGOT

Alltech Inc., Nicholasville, Kentucky, USA

Global molasses production increasing

World molasses production has increased markedly over the past 40 years from around 15 million mT in the early 1960s to around 45 million mT by 2000 (Anon., 2002, Table 1). While intricately tied to the production of rum in the Caribbean region, sugar crops are also the feedstock for over 60% of world fuel ethanol production (Berg, 1999). Around 75% of the world's molasses comes from sugar cane (*Saccharum officinarum*) grown in tropical climates (Asia, South America), while the remainder comes primarily from sugar beet (*Beta vulgaris*) grown in the more temperate climates of Europe and North America.

Table 1. World molasses production by region.

	*2002/03**	*2001/02*	*2000/01*
		1000 mT	
EU	3,950	3,650	3,966
Western Europe	4,800	4,359	4,856
Eastern Europe	2,420	2,391	2,224
Africa	3,340	3,307	3,260
North/Central America	6,830	6,677	6,874
South America	12,900	11,626	10,182
Asia	18,120	18,019	16,511
Oceania	1,080	1,023	1,030
World	49,490	47,402	44,957

Anon., 2002
*Predicted

The perfect feedstock?

At first sight molasses appears to be the perfect feedstock for production of alcohol. All the sugars are present in a readily fermentable form. Unlike grains, which contain starch instead of sugar, molasses need not be cooked or processed. It is also in liquid form, which makes it easily handled. However the reality is that molasses has a range of problems and quirks that make it far from a perfect feedstock, and knowledge of its composition and characteristics are very important if it is intended as a substrate for alcohol production.

Molasses production

There are several types of molasses (Table 2) defined by sources and process, however the information in this chapter will focus on the molasses produced as a by-product of sugar production from cane and beets. Cane or beet molasses is the end product after the sugar has been removed by crystallization. Cane sugar is produced by first heating the juice crushed from cane. It is then clarified by filtration with lime added to precipitate fiber and sludge. The slurry is then evaporated to concentrate the sugar and allow it to crystallize. The syrup containing the crystals is then centrifuged to separate the crystals and the syrup residue. This first residue, which still has a high content of sugar, is referred to as 'A molasses'. The process is repeated to yield more sugar and subsequent B, C, or even D molasses grades. Sugar mills normally evaporate and centrifuge a maximum of three times, but the number of treatments will depend on marketplace economics. Obviously successive crystallizations reduce the amount of sugar present and lessen its value as a fermentable substrate for ethanol production. Repeated crystallization also tends to degrade molasses sugars to unfermentable compounds (Murtagh, 1999).

Table 2. Types of molasses.

Blackstrap molasses	By-product of sugar production from sugar cane
High test (cane) molasses	Primary product: extracted from sugar cane
Refiners cane or beet molasses	By-product of refining raw brown sugar to produce white sugar
Beet molasses	By-product of sugar production from sugar beets
Citrus molasses	Juices extracted from the manufacture of dried citrus pulp

BLACKSTRAP MOLASSES

Blackstrap (cane) molasses is used in animal feeds and is also the most common feedstock when using molasses to make alcohol. Since it is a waste product and a commodity typically gathered from many plants, unless the source is known it can be expected to vary considerably. Even from the same plant, different operating conditions can produce a product that ferments very differently from batch to batch.

HIGH-TEST MOLASSES

In contrast to other types of molasses, high-test molasses is not a by-product. It is produced *instead* of sugar as opposed to being a by-product of sugar production. It is clear, light brown and of controlled composition. The process for making high test molasses is exactly the same as the production of the sugar, however, the sugar cane syrup is instead just concentrated, partially inverted using either invertase from *Saccharomyces cerevisiae* or acid (not favored as it destroys about 5% of sugars) to prevent crystallization in the final product. Apart from the fact that high test molasses is more expensive, this product has several advantages over typical molasses as a feedstock for the production of ethanol. It is lower in ash, has fewer dissolved impurities and has a more accurate and controlled sugar profile and sugar content. Typical composition is summarized in Table 3.

Table 3. Typical composition of high test molasses.

°Brix	85.0
Sucrose, %	27.0
Reducing sugars, %	50.0
Calcium, %	0.2
Ash, %	2.25
Water, %	15.50

Chen and Chou, 2003

CANE VS. BEET MOLASSES

The molasses obtained from cane and beet sugar production are similar but not identical. Molasses produced from beets cannot legally be used for rum and beverage produced from beet molasses has an off flavor and character. Beet molasses is also slightly lower in sugars than cane molasses; and its sugar profile consists almost entirely of sucrose (Table 4). Organic acid types and amounts in beet molasses vary considerably, as does the ash content. The content of other minerals is slightly lower in beet than in cane molasses. The pH of beet molasses is slightly more neutral (around pH 7) than cane molasses, which is typically pH 5 to 5.5.

Since cane and beet molasses are agricultural products, chemical composition will be affected to varying extents by climatic conditions, soil, fertilization practices, moisture, etc., as well as the method of production and sugar removal from the product. This also means that molasses composition can vary greatly among refineries or even from the same refinery during a single crushing season.

Table 4. Comparative chemical composition of beet and cane molasses.

	Cane	*Beet*
	Range or typical value (%)	
Total sugars	45-55	48-52
Sucrose	25 to 35	46-52
Reducing sugars	20 to 35	0.5-4.0
pH	5-5.5	~7.0
Ash	10-16	10-12
Non-sugar organic matter	10-12	12-17
Starch/polysaccharides	0.5%	
Calcium	0.4-0.8	0.1-0.5
Sodium	0.1-0.4	0.5-1.0
Potassium	1.5-5.0	2.0-7.0
Magnesium	0.05-0.98	0.1-0.15
Phosphorous	0.03-0.1	0.02-0.07
Sulfur	0.3-0.8	0.15-0.5
	Range or typical value (ppm)	
Biotin	1.2-3.2	0.04-0.13
Folic acid	0.04	0.2
Riboflavin	2.5	0.4
Copper	2.2-38	2.5
Manganese	4-300	3.0
Zinc	4-48	0.15

United Molasses

Basic sugar chemistry helpful in understanding molasses as a substrate

SUCROSE STRUCTURE AND HYDROLYSIS

Sucrose, common table sugar, is a disaccharide composed of one glucose and one fructose molecule. Sucrose is the principal sugar in molasses. On hydrolysis (reaction with water), sucrose yields the two monosaccharides (Figure 1). It should be noted that the molecular weight of sucrose is less than the total of its component monosaccharides because a molecule of water is displaced from sucrose and transferred to the simple sugars.

INVERT AND REDUCING SUGARS, SUGAR MEASUREMENTS IN MOLASSES

In aqueous solutions, monosaccharides containing five or more carbons occur in cyclic forms. These pyranose (6-carbon) or furanose (5-carbon) rings form when the aldehyde (or ketone) group reacts with a hydroxyl group at the other end of the molecule to form a hemiacetal (or hemiketal) linkage. Another chiral center is produced, known as the anomeric carbon (Figure 2). The hydroxyl group (OH) can be either above the plane of the ring (ß-glucose), or below the plane (α-glucose). α- and ß-glucose are known as anomers, because they differ only in the position of the group at the anomeric carbon.

It is this free anomeric carbon that makes glucose and fructose *reducing sugars*, which refers to their ability to reduce a standard copper sulfate solution such as Fehling's solution. Sucrose does not have this property. The two monosaccharides are also referred to as *invert sugar*. This is derived from the fact that sucrose rotates polarized light to the right (dextrorotary), while the two hydrolysis products rotate polarized light to the left (levorotatory). During

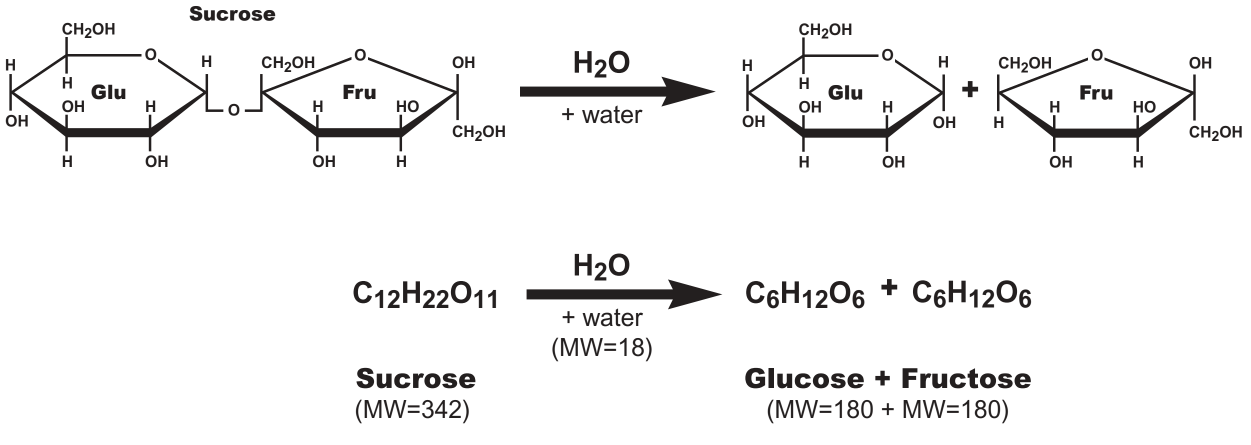


Figure 1. Hydrolysis of sucrose.

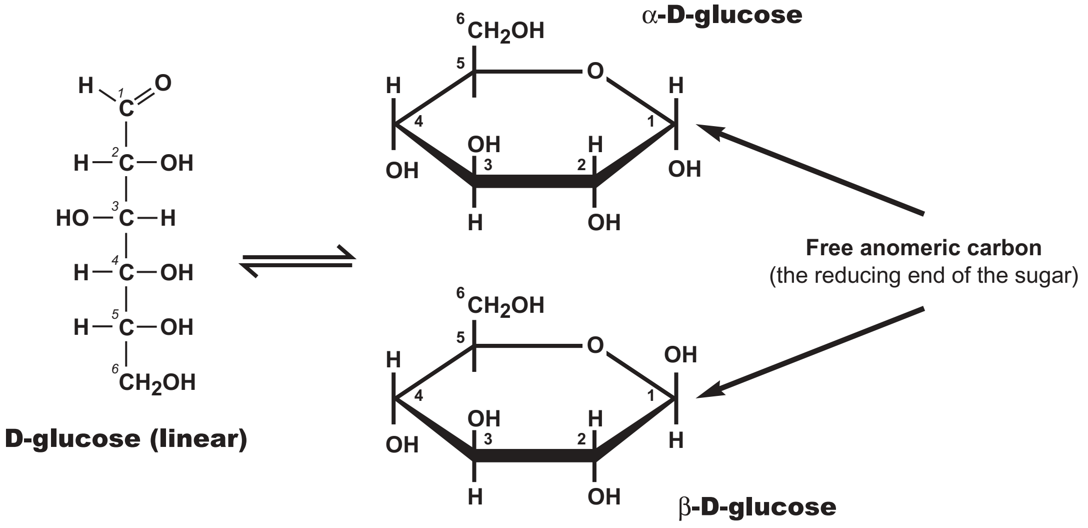


Figure 2. Linear and ring structures of glucose.

fermentation yeast produces an enzyme (invertase) that breaks sucrose down into invert sugars.

Total sugars as invert (TSAI) is a measurement commonly used to gauge molasses sugar content. This is either a calculated value or based on reducing power of a sample, which is at best a crude measurement. Measuring reducing power assumes that all the reducing compounds in molasses are sugars. Thus any sodium sulfite added would appear as sugar in such a test. There is often up to 5% difference in this test method over the actual amount of reducing sugars present. Sugar measurements for molasses are summarized in Table 5.

DEXTRANS

Dextrans are sucrose molecules that have polymerized into long chains. This polymerization can occur when molasses has been stored for a long period of time or if it has been contaminated with *Leuconostoc mesenteroides*. While dextrans appear as reducing sugars, they are not fermentable. A bad contamination will result in molasses having long 'strings' or a ropey appearance caused by dextran formation. A recent development by Alltech has been use of dextranase (used at a rate of 0.01% by weight of molasses) to hydrolyze the dextran chains.

Both sucrose and its component monosaccharides are readily fermented by yeast.

BRIX VALUES

Repeated evaporation and centrifugation increase molasses viscosity and concentrates salts and other impurities to form a thick, viscous, brown liquid. The concentration of molasses is normally measured in degrees Brix. The Brix

Table 5. Measurements of molasses sugar content used in trade.

Term	*Description/calculation*
TSAI (Total sugars as invert)	(Sucrose (%)/0.95) + invert sugars (%)
TSM (Total sugars)	Sucrose (%) + invert sugars (%)
TSAS (Total sugars and sucrose)	Sucrose plus the amount of invert sugar converted to sucrose: sucrose (%) + (invert (%) x 0.95)
Brix	The sugar content of a solution with the same specific gravity of a molasses sample (same as the Balling scale). Measured using either a refractometer or hydrometer.

scale is the same as the Balling scale, which is a measure of what the sugar content of a liquid would be if all the dissolved and suspended solids were sugar. Expressed another way, it is the sugar content of a sugar solution with the same specific gravity as the sample. Thus, an 80°Brix molasses has a specific gravity of 1.416, which is the same as a sugar solution containing 80% sugar by weight. Brix is measured using a hydrometer originally intended only for sugar solutions. Unfortunately with molasses, the presence of other dissolved solids means that Brix readings often bear little relationship to the amount of sugar actually present. Despite this, Brix values are quite commonly used in the sugar industry; and molasses is often defined as around 80°Brix. Brix values determined by refractometer and hydrometer rarely agree, so the method by which the reading was obtained should be clearly stated.

Molasses handling and storage

Molasses is generally quite stable and can be stored for long periods of time if properly managed. That said, a 2.3 million gallon tank of molasses measuring 90 ft in diameter and 52 ft high exploded in Boston in 1919 killing 21 people. The 'wall of goo' smashed buildings and knocked out stone supports for an elevated train. Needless to say it took months to clean up, and the distillery was fined extensively for damages. Paradoxically, this odd, but true urban legend occurred in January when temperatures warmed to an unseasonable 46°F. Clearly proper design and temperature control are important for molasses storage tanks. It can be stored in either steel tanks or underground concrete tanks. Both are very satisfactory, however, it is critically important that the molasses is kept above 70°Brix. This means it must be kept out of the rain and free from any condensation inside the storage vessel. The storage should be well ventilated. If moisture collects even in small pools on the surface, then mold and other organisms held in check by the high sugar content in the molasses will flourish.

During processing in the sugar refinery and during handling, air can become entrained in the molasses. In addition, carbon dioxide is evolved during molasses degradation. Carbon dioxide bubbles rise very slowly in the molasses, meaning that density at various levels in a storage tank will be quite different. This makes it impossible to calculate with precision from a molasses sample taken from a single point in the tank and a measure of the liquid height to tell exactly how much molasses is present in the tank. The way to more accurately measure the amount of molasses present is to take a reading with a device such as a pressure cell that will measure the actual weight of the molasses in the tank. This will not significantly vary with the amount of entrained air. It also means that production procedures are more consistent in that it is possible to know exactly how much molasses has been processed.

VISCOSITY: DILUTING MOLASSES

Molasses is well known for being viscous especially at lower temperatures. Molasses viscosity varies widely, depending on several factors including dry solids content. Some equipment is designed to take advantage of this viscosity to help move the molasses. As a general rule, pumps should be positive displacement with inlets as large (at least 8 to 10 cm) and as direct as possible. The pump should have large inlet cavities and turn as slowly as possible to allow time for the molasses to properly fill the chamber. A pump turning too quickly will cavitate.

Dilution will assist in movement; and dilution to 40°Brix is typical. Dilution should occur just prior to the fermentation or clarification/pasteurization process, however, as microbes can start to grow at the reduced osmotic pressure caused by dilution.

When diluting molasses, it must be remembered that the Brix scale measures on a weight % basis and all calculations must be based on weight and not volume. 80°Brix molasses has a specific gravity of 1.416, therefore a gallon weighs about 11.8 lbs and a ton contains about 169.5 gallons. Example calculations for diluting 80°Brix molasses to 40° and 25°Brix are given in Table 6. Temperature of the water and molasses will have a large impact on these calculations (Figure 3).

Table 6. Molasses dilution: dilute 1 ton (or tonne) of 80ºBrix molasses to 25ºBrix.

	Gallons	*Liters*
Total tons: 80/25 = 3.2 tons (or tonnes) at 25ºBrix		
Volume required at 25ºBrix		
Molasses (1 ton or tonne at 80ºBrix*)	169.5	706
Water (2.2 tons or tonnes)	528	2,200
Total volume produced at 25ºBrix	697.5	2906

*assuming 80ºBrix molasses has a specific gravity of 1.416 at 20°C.

DEGRADATION DURING STORAGE AND PROCESSING

As illustrated in Figure 4, heating will lower viscosity; however little is gained by temperatures above 35°C and it is advised to keep temperature below 40°C to reduce thermal degradation (Figure 5). If the molasses is to be heated in a tank it is recommended to use a coil hot water system rather than steam. Heating should be done gently to avoid spot heating. Three hours at 120°C can reduce sugar content by as much as 10 to 15%. At 130°C sugar loss increases to 25-35% (United Molasses).

Fermentation

Molasses makes a good base for fermentation. In fact, most of the yeast produced in the world is made using a molasses substrate. However there are several potential problems with molasses that must be considered if fermentations are to be efficient and cost-effective.

The amount of fermentable sugar varies greatly in molasses. In order to standardize, yield calculations are usually based on 55% sugars. A good fermentation with high quality molasses should yield about 290 liters of ethanol per ton of molasses. However recent molasses shipments have yielded more on the order of 260 liters/tonne. Molasses prices are set internationally; and typically run $USD35-40 per ton FOB with freight added raw material cost per liter of alcohol can be $0.20-0.25.

With the increasing efficiency of the sugar industry in extracting sugar, the molasses remaining for use in fermentation/distillation operations is becoming poorer in quality. Some plants are finding it necessary to sterilize and

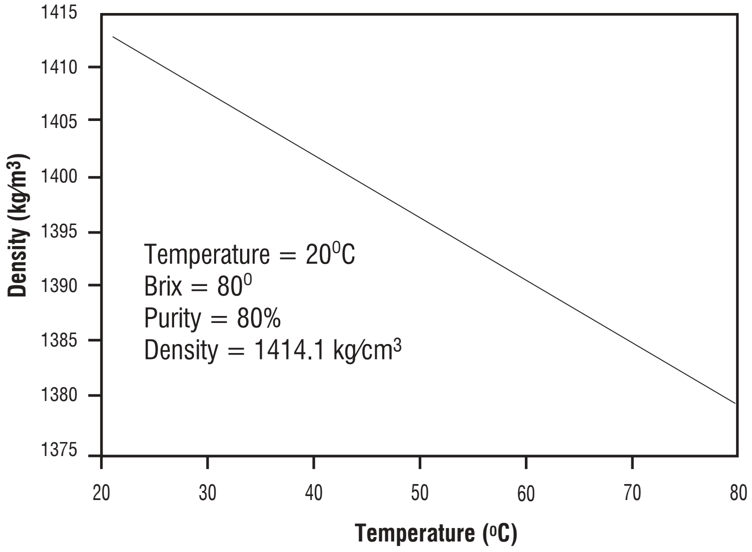


Figure 3. Effect of temperature on density of molasses.

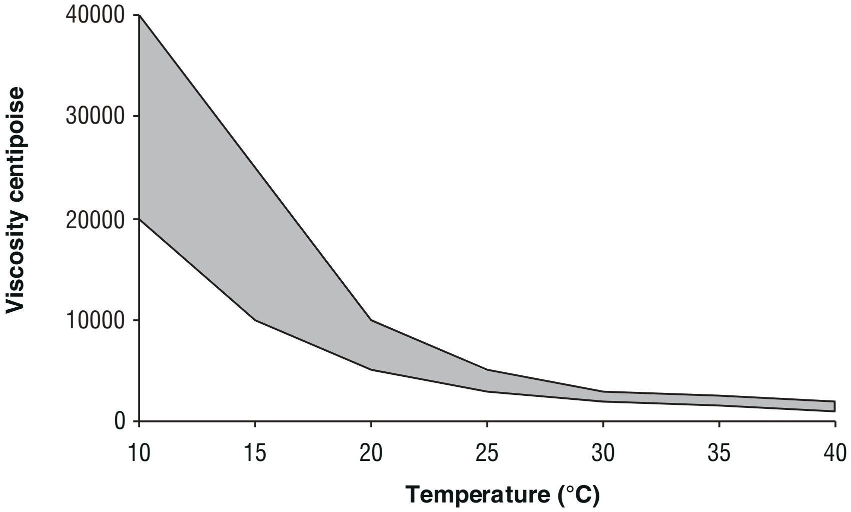


Figure 4. Effects of temperature on the viscosity of cane molasses (R&H Hall, 1999).

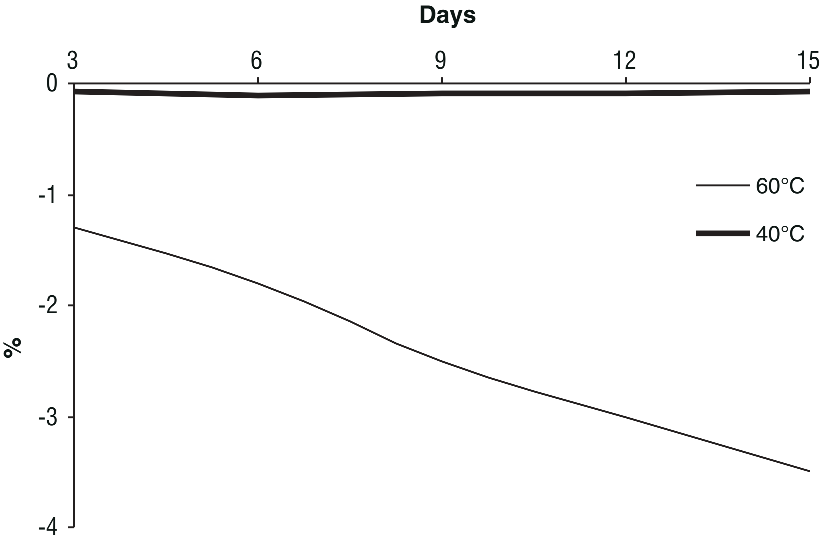


Figure 5. Sugar losses in molasses stored at 60 or 40°C over 15 days (United Molasses).

clarify before fermentation, while others employ dextranase to ensure maximum extraction of whatever sugar is present. This is accomplished by diluting the molasses to approximately 40°Brix, then heating it. Gums and other suspended solids drop out of the diluted solution. Following heating to pasteurization temperature for an appropriate time the molasses is cooled and sent to fermentation. The disadvantage of this process is the energy input required as well as some loss of sugar.

Molasses at 80ºBrix will not ferment without dilution as the sugars and salts exert a very high osmotic pressure. It is therefore necessary to dilute the molasses to below 25ºBrix. Yeast will not start fermenting rapidly above this point; and contamination may develop before the yeast become established since molasses is laden with contaminating bacteria. Some distilleries acidify the wash to help reduce infection. Sulfate added at this point can come out later as calcium sulfate scale in the wash column.

Brix values of about 25° are the highest osmotic pressure that yeast will tolerate to begin fermentation, however this means a low sugar content and potential low fermentor alcohol levels. If sugar content is initially 46%, then dilution to 25°Brix results in only ~14.3% sugar

(Calculated: 25 x 46/80 = 14.3). This results in about 8% alcohol. This is low by today's standards and results in high hydraulic loading and large volumes of effluent. One way to increase the alcohol content of the fermentation is by incremental feeding. After the fermentation has been going several hours and has dropped to 15 or 16°Brix, additional molasses (at 40°Brix) is added to bring concentration to 20°Brix and this mixture is then fermented. This allows yeast to become rapidly established.

YEAST AND YEAST CONDITIONING

It is important that very high quality yeast are added at high cell counts to the fermentation. Unlike grain fermentations where the sugar or glucose is produced throughout fermentation, all the fermentable substrate in molasses is present at the beginning of fermentation and it is equally available to yeast and any undesirable contaminants that might be present. In the battle determining what species is to dominate fermentation, healthy yeast are critical. Many distilleries condition the yeast before adding it to fermentation. This is done by using ~10% of the final fermentor volume diluted to about 16°Brix. Yeast is added, as is a small amount of air, yeast food (eg, AYF™ at 10-15 ppm) and possibly antibiotics to ensure that other organisms are not being grown. A low pH and a temperature around 28°C also help keep contamination in check. It is important that conditioning tanks are kept clean and that yeast is pumped to the fermentor while still in the exponential growth phase.

Molasses fermentations are very rapid, often completed in 24 hrs, and so much heat is generated that a good cooling system is very important. Since many of these fermentation systems are located in tropical regions where cooling is limited, a temperature tolerant yeast strain can be of great value. Thermosacc™, a high temperature yeast (97-100°F) designed originally for grain distilleries, is now finding application in this industry.

Yeast nutrition

The free amino nitrogen (FAN) available in molasses varies widely for a number of reasons, including variable amounts in the original syrup and/or losses during during processing and storage. Unless FAN can be tested on a very regular basis it is recommended that a sufficient source of FAN is added to ensure good fermentations. Nitrogen should not be in the form of ammonium sulfate as it will add to the scaling problem by forming calcium sulfate. Likewise, liquid ammonia is undesirable as it tends to raise the pH and encourage bacterial contamination unless it is counteracted with acid additions. (Some plants using liquid ammonia either use sulfuric acid to balance the pH, which introduces the undesirable sulfate anion, or use phosphoric acid, which generally introduces more phosphate than necessary).

Urea may be used to supply nitrogen in molasses fermentations for fuel ethanol production, but its use for beverage alcohol production should be approached with caution. Urea usage may lead to the production of carcinogenic ethyl carbamate, which is unacceptable in alcoholic beverages. If phosphorus is deficient in the molasses, diammonium phosphate may be added with a corresponding reduction in urea or other nitrogenous nutrient. Generally, blackstrap molasses requires no other added nutrients for fermentation.

The three B vitamins most likely to be essential for the satisfactory growth of any particular strain of *Saccharomyces cerevisiae* yeast are biotin, pantothenic acid (pantothenate) and inositol. Biotin is normally present in great excess in cane molasses, but is normally deficient in beet molasses. If a yeast strain that does not require biotin cannot be obtained (they are rare), it may be advantageous to mix in 20% cane molasses when trying to ferment beet molasses, or at least to use a 50:50 cane:beet molasses mix for initial propagation of the yeast.

Pantothenate is normally borderline-to-adequate in cane molasses, but is generally in excess in beet molasses. Mixing some beet molasses with blackstrap cane molasses may be advantageous if it is available. Pantothenate concentrations may also be low in HTM and in refiners cane molasses (the by-product of refining raw brown sugar to produce white sugar). In these instances, it may help to add some beet molasses to the mash although one may obtain yeasts that do not require external sources of pantothenate for growth.

Inositol may be deficient in HTM, but this deficiency is less critical as it is relatively easy to find yeasts that do not require external sources of inositol.

CONTAMINATION OF MOLASSES FERMENTATIONS

The principal contaminant of molasses fermentations is *Leuconostoc mesenteroides*, which causes sucrose molecules to polymerize into unfermentable dextran chains. If the contamination is very extensive, the molasses may appear to be 'ropey'. Dextran may also be found in refiners molasses produced from raw sugar that has been stockpiled for a number of years. Apart from the loss of yield, the presence of dextran increases foaming in fermentors.

Another important occasional bacterial contaminant is *Zymomonas mobilis*. This bacteria can ferment sugars to ethanol, but has the side effect of reducing sulfur compounds to produce a hydrogen sulfide smell, which is very undesirable for production of good quality rum.

Distillation

There are several issues that are somewhat unique to the distillation of molasses. Scaling in the wash column is a serious problem. It is largely caused by lime used in the clarification process at the refinery. The most common ways to combat this are to use a non-fouling tray design and/or run the column under vacuum to keep the temperature down and reduce scale formation. Additives can be added to reduce scaling and special care should be taken to keep the alcohol percentage on the feed tray as low as possible. High strength spirit on this tray will increase scale accumulation.

Mud, or sludge, often settles at the bottom of the fermentor. If this occurs, then wash can be drawn off at a point above the fermentor base. Sludge that has settled in the bottom of the fermentor can be discarded separately. A non-fouling tray should be able to handle these solids and allow them to pass out in the dunder. If not, then evaporator fouling can occur when evaporating the stillage to produce condensed molasses solubles (CMS).

Molasses fermentations have a tendency to be high in sulfur. This is especially true if the fermentation is infected with *Zymomonas mobilis*.

Methanol is not common in molasses fermentation, because molasses contains very little pectin which is broken down in some feedstocks during fermentation to form methanol. Hence the amount of methanol in molasses spirit is low.

Effluent treatment

The liquid effluent from the rum production process (known as spent wash, stillage, vinasse or dunder) is a significant environmental problem. Biological Oxygen Demand (BOD) is in the range of 50,000 mg/l and the effluent is very dark in color. There is no 100% effective method of treatment available; and no single treatment would suit all distilleries.

At present there are six primary ways that molasses effluent is treated.

1. Dilution and discharge into the sea
2. Land application
3. Deep well injection
4. Anaerobic digestion prior to one of the above
5. Lagoon treatment prior to one of the above
6. Condensation to CMS then used as a cattle food supplement or incinerated

DILUTION AND DISCHARGE INTO THE SEA

This is a controversial method of disposal, but is used by several distilleries. It has been studied in at least two cases and found not to be harmful to the environment if done properly. The BOD dissipates rapidly, but the color change can be visible for miles from the outlet. This is both visibly unappealing and it also blocks light from penetrating the water. In areas of the Caribbean where tourism is a major industry, foul smells and brown seawater are not acceptable.

LAND APPLICATION

Stillage can be applied to the soil surface and incorporated into the top 15 to 30 cm in three different ways:

Soil conditioner. This approach takes advantage of the available organic and inorganic nutrients in effluent to enhance the growth of certain crops. This is a good option when a distillery is located in an area with the correct soil type and crops. Unfortunately this is often not the case.

Compost/soil improvement. In land reclamation, or soil improvement to add organic and inorganic nutrients to marginal lands.

Disposal site. On a plot of land set aside for the purpose of disposal, whereby the organic constituents are degraded by natural processes (oxidation and reduction via sunlight) and the inorganic components are trapped within the soil matrix.

DEEP WELL INJECTION

Injection of stillage in a deep well is one method of land application employed, but has caused ground water contamination in some areas.

ANAEROBIC BIODIGESTERS

Bacardi have done a considerable amount of work on anaerobic biodigesters. This process reduces BOD by about 80% but has little effect on effluent color. In order to reduce the BOD to acceptable levels and reduce color, an aerobic finishing step may be required. This leaves a sludge for disposal. This sludge may have some use as an animal feed supplement. The methane gas from the anaerobic digester can be used to run a boiler in the distillery. The author's experience with this process is that it works well, but the value of the captured gas is not much more than the cost of operating the process, which results in a long capital payback.

LAGOON

Effluent held in a lagoon is sometimes treated with lime to adjust pH. Other types of treatment such as aeration, enzyme and/or bacterial additives, have been used in a few distilleries with apparent success.

EVAPORATION TO CONDENSED MOLASSES SOLUBLES

Cane molasses stillage can be evaporated to about 50°Brix and sold as an animal feed supplement. Its comparatively low value makes this option more of a 'break even' prospect depending on the cost of energy to run the evaporators. While CMS can be incinerated, additional cost is incurred. Use as a fertilizer is another option, however demand is limited.

References

Anon. 2002. Third estimate of world molasses production 2001/02. F.O. Licht's World Ethanol & Biofuels Report. July 15.

Berg, C. 1999. World Ethanol Production and Trade to 2000 and Beyond. F.O. Licht. 80 Calverley Road, Tunbridge Wells, Kent, TN1 2UN, England.

Chen, J.C. and C.C. Chou. 2003. Cane Sugar Handbook: A Manual for Cane Sugar Manufacturers and Their Chemists. John Wiley & Sons, New Jersey.

Murtaugh, J.E. 1999. Molasses as a feedstock for alcohol production. In: *The Alcohol Textbook, 3rd Ed.* (K.A. Jacques, T.P. Lyons and D.R. Kelsall, eds). Nottingham University Press, UK.

R&H Hall. 1999. Molasses in livestock feeds. Technical Bulletin No. 3. *http://www.rhhall.ie/print/issue3_1999.html.*

United Molasses.

Yeast and management of fermentation

Chapter 9

Understanding yeast fundamentals

INGE RUSSELL

International Centre for Brewing and Distilling, School of Life Sciences, Heriot-Watt University, Edinburgh, UK

Introduction

The heart of the fermentation process is the yeast cell. Yeast is the least expensive raw material input into the fermentation process whether it is for beer, distilled spirits or fuel ethanol, and yet too often the importance of the yeast is undervalued. The yeast is ignored, abused and economized. A healthy and well-selected yeast is needed for an efficient fermentation. When selecting the strain, attention must be paid to whether a yeast is needed that can function at low temperatures such as for lager beer production or at high temperatures for fuel ethanol production. Whether the environment requires a yeast with the ability to survive high ethanol concentrations, or whether the flavor compounds that the yeast produces are of interest or concern. The yeast is an amazingly hardy organism that can survive under many stressful conditions, but under stress it will not be working at its optimum. A careful choice as to the particular strain and meticulous attention to growth conditions will lead to successful fermentations.

Traditionally yeast have been characterized according to morphological and physiological criteria. Taxonomists have grouped together brewing (both ale and lager), distilling, wine, baking, and numerous wild yeasts, under the name *Saccharomyces cerevisiae*. This chapter will only deal with *Saccharomyces* yeast, specifically *S. cerevisiae* unless otherwise noted. This is the microorganism used globally for potable and industrial ethanol production. Ethanol is the largest commodity produced by any microorganism; and world ethanol production in calendar year 2003 is expected to increase to 38 billion liters/year from 34.3 billion in 2002.

What is a yeast?

A common definition is *'yeast is a fungus where the unicellular form is the most predominant and which reproduces by budding (or fission)'*. The term yeast is often used synonymously with *Saccharomyces cerevisiae* since this is the species that is produced in the largest amounts for both baking and fermentation purposes. *Saccharomyces* yeast reproduces *only* by budding. Yeast are considerably larger in size than bacteria and in terms of quantity and economics are the most important group of microorganisms exploited for industrial purposes. Over 700 different species of yeast have been identified by taxonomists. Many are poorly characterized, but the ones of industrial importance have been well characterized. Indeed *Saccharomyces*, of which there are thousands of unique strains, was one of the first organisms to have its entire genome sequenced and available on the internet.

Why is a yeast so effective at fermentation?

Yeast is unique in that it is the only living microorganism that can switch from respiration to fermentation. An important fact to remember about yeast is that despite the presence of oxygen, yeast will always take the fermentative route to utilize glucose if the glucose is there in high concentration (called the *Crabtree effect*).

The world of yeast is very small and we measure this microscopic universe in terms of the Greek letter μ for micron (a micron being 10^{-6} meter). A single yeast cell measuring 7 μm x 7 μm would have a surface area of 153 μm^2. Ten grams of pressed yeast would have a contact surface area of ~10 m^2. It is this large contact surface area that allows yeast to so effectively take up sugars from the surrounding fermentation medium. A useful rule of thumb is that one gram dryweight of yeast equates to approximately 4.87 x 10^{10} cells.

Strictly speaking, *Saccharomyces* cannot be considered a true facultative anaerobe since the Crabtree effect takes precedence, i.e. above 1% fermentable sugar in the culture medium (the exact percentage varying between strains) the yeast ferments the sugar by anaerobic metabolism, despite how well the culture medium is aerated.

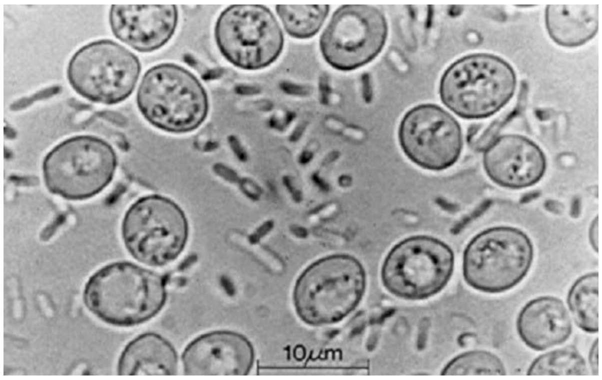


Figure 1. Yeast and bacteria.

The main parts of a yeast cell

Yeast cells vary from oval to round and in size from roughly 5 to 10 μm length to a breadth of 5 to 7 μm, but there are exceptions ranging from 2.5 to 21 μm. Yeast are roughly the size of a red blood cell and many times larger than a bacterial cell (Figures 1 and 2). The mean cell size varies with the growth cycle stage, the growth conditions and the age of the cell (older cells becoming larger in size). The main components of the yeast cell are illustrated in Figure 3.

Under the microscope one can see the yeast cell wall along with multiple bud scars and a birth scar (Figure 4). The cell wall gives shape and stability as well as cell-to-cell recognition. It acts as a permeability barrier to large solutes and plays a role in controlling the entry of water into the cell. The cell wall also protects the yeast cell membrane. The yeast cell wall is composed

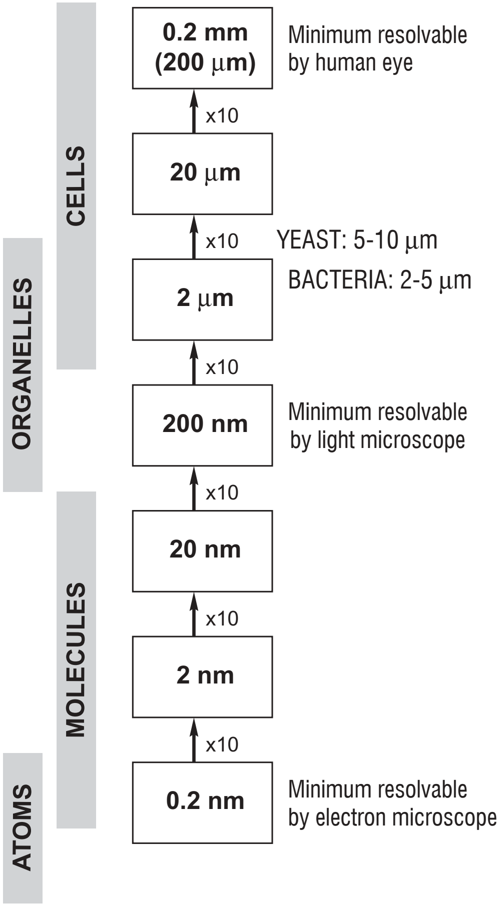


Figure 2. Relative sizes of yeast and bacteria.

primarily of mannan and glucan (Figure 5). The yeast cell plasma membrane is primarily lipids and proteins with a small amount of carbohydrate and acts as the barrier between the cytoplasm and the outside of the cell. It is very important as it regulates what goes in and out of the cell (Figure 6).

The cell nucleus consists primarily of DNA and protein and is surrounded by a nuclear membrane. The nucleus can be seen using phase contrast microscopy. The periplasmic space is the area between the cell wall and the cell membrane. It contains secreted proteins that are unable to cross the cell wall. For example, some enzymes located here help in the catalysis of sugars such as sucrose. Sucrose is broken down in the periplasmic space by the enzyme invertase, to fructose and glucose.

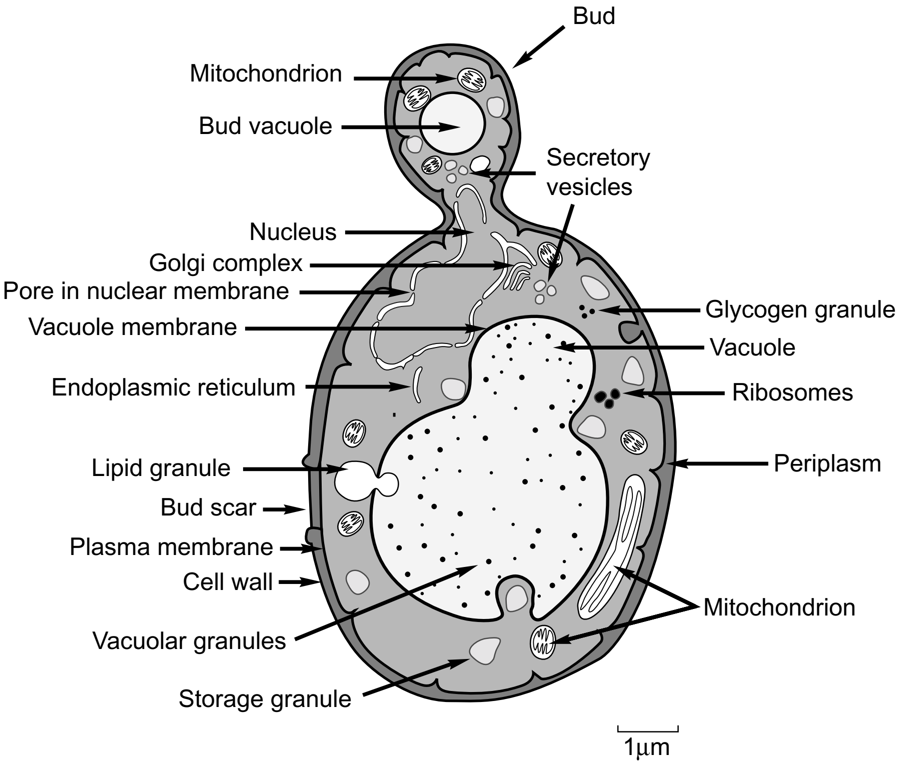

Bud
Mitochondrion
Bud vacuole
Secretory vesicles
Nucleus
Golgi complex
Pore in nuclear membrane
Vacuole membrane
Glycogen granule
Vacuole
Endoplasmic reticulum
Ribosomes
Lipid granule
Periplasm
Bud scar
Plasma membrane
Cell wall
Mitochondrion
Vacuolar granules
Storage granule
1µm

Figure 3. The main features of a typical yeast cell.

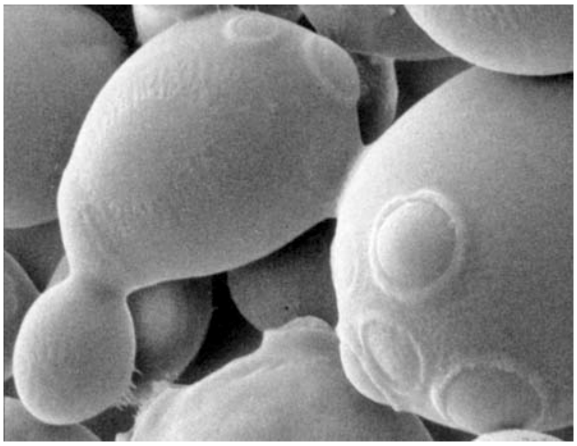


Figure 4. Electron micrograph of a yeast cell with multiple bud scars (and birth scar).

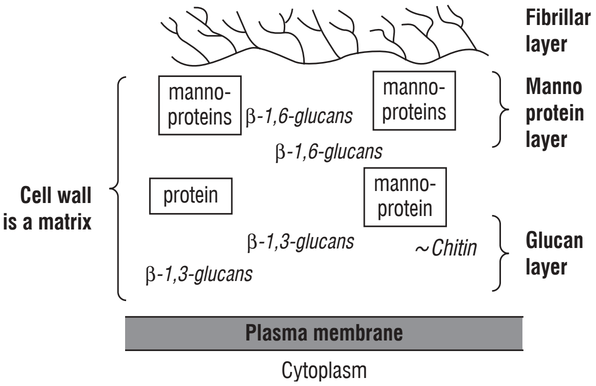


Figure 5. Structure of the yeast cell wall.

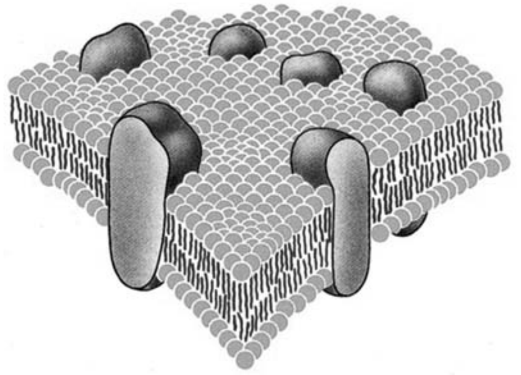


Figure 6. Structure of the plasma membrane.

The mitochondria can only be seen using electron microscopy and consist of structures with two distinct membranes, the outer and the inner, and cristae within the mitochondria are formed by the folding of the inner membrane. Mitochondria are of importance since it is here that the enzymes involved in the tricarboxylic acid (TCA or Krebs) cycle and in electron transport and oxidative phosphorylation are located. Mitochondria contain self-replicating DNA and protein synthesis systems. Ribosomes, the site of protein synthesis, are located in the cytoplasm. Vacuoles are part of the intramembranous system, which includes the endoplasmic reticulum. The size and shape of the vacuoles change during the cell cycle and vacuoles are the most obvious component of the cell when viewed under the light microscope. The vacuoles store nutrients and provide a location for the breakdown of macromolecules such as protein. Lipid granules are found in the cytoplasm, as are high concentrations of glycogen. Glycogen can be visualized under the light microscope by staining the cells with iodine.

The lifespan of yeast

Individual yeast cells are mortal. Yeast aging is a function of the number of divisions undertaken by an individual cell and not a function of the cell's chronological age. Lifespan is measured by counting the bud scars on the surface of the cell. All yeast have a set lifespan determined both by genetics and environment. The maximum division capacity of a cell is called the *Hayflick Limit*. Once cells reach this limit they cannot replicate and enter a stage called senescence, which leads to cell death. This is particularly important with respect to recycling yeast, which cannot be done without loss of some metabolic function. A new yeast cell (daughter or bud) can divide to produce 10 to 33 daughters of its own depending on the strain. Industrial polyploid ale yeast strains have been reported to have a lifespan varying from a high of 21.7 +/- 7.5 divisions to a low of 10.3 +/- 4.7 divisions. Respiratory deficient (petite) mutants of these strains were shown to have a reduced lifespan. When examining yeast under the microscope the following are signs of aging: an increase in bud scar number, cell size, surface wrinkles, granularity, and daughter cells being retained. Aging is accompanied by a progressive impairment of cellular mechanisms that eventually results in a yeast with a reduced capacity to adapt to stress and to ferment (Smart, 2000). Figure 7 shows the progression through the lifespan cycle of a yeast cell.

The chemical composition of yeast

A yeast cell consists of 75% water and 25% dry matter. The dry matter consists of:

Carbohydrate	18-44%
Protein	36-60%
Nucleic acids	4-8%
Lipids	4-7%
Total inorganics	6-10%
Phosphorus	1-3%
Potassium	1-3%
Sulfur	0.4%
Vitamins	trace amounts

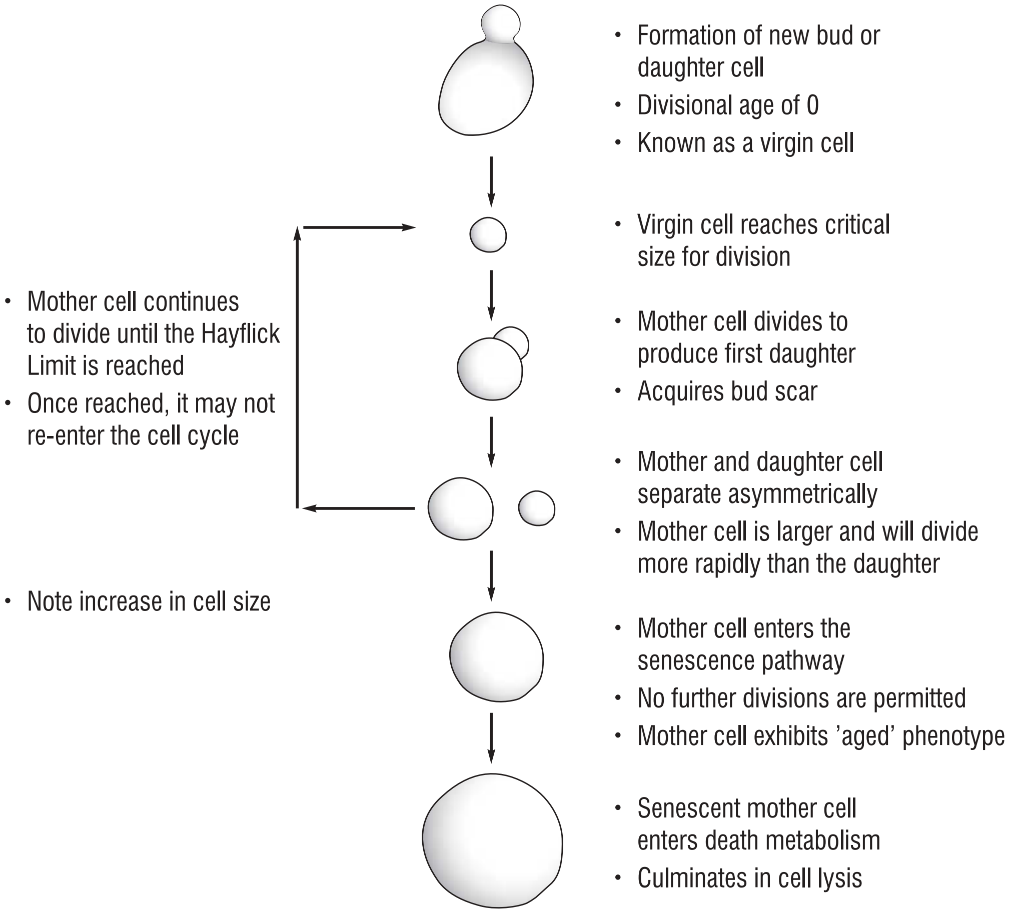

• Formation of new bud or daughter cell
• Divisional age of 0
• Known as a virgin cell
• Virgin cell reaches critical size for division
• Mother cell continues to divide until the Hayflick Limit is reached
• Once reached, it may not re-enter the cell cycle
• Mother cell divides to produce first daughter
• Acquires bud scar
• Mother and daughter cell separate asymmetrically
• Mother cell is larger and will divide more rapidly than the daughter
• Note increase in cell size
• Mother cell enters the senescence pathway
• No further divisions are permitted
• Mother cell exhibits 'aged' phenotype
• Senescent mother cell enters death metabolism
• Culminates in cell lysis

Figure 7. The lifespan of yeast (adapted from Smart, 2000).

When a sample of yeast is analyzed, its composition will depend greatly on the genotype of the yeast as well as the growth parameters and hence the differences in composition between a yeast propagated for the baking industry *vs* a yeast propagated for use in the fermentation industry. Table 1, compiled from a number of sources, gives a good indication as to the constituents of a typical yeast cell. The exact values will vary with the particular strains examined, the composition of the media, as well as many environmental factors. The general composition of yeast guides us to the levels of nutrients that the yeast may require in order to grow.

Yeast growth requirements

Specific requirements for yeast growth include:

i) water

ii) a carbon source - fermentable carbohydrates as an energy source

iii) oxygen/lipids - lipids for membrane biosynthesis, which can be made by the yeast if oxygen is present

iv) a nitrogen source - amino acids and peptides are needed for growth and enzyme synthesis. *Saccharomyces* yeast can also use ammonia.

v) growth factors -vitamins

vi) inorganic ions - essential for yeast metabolism

WATER

Water is a main component of all living material and most microorganisms need at least 15% water

Table 1. Approximate composition of industrially produced aerobically grown baker's and anaerobically grown brewer's yeast (expressed as g/kg dry yeast) (from Ingledew, 1999).

Cell composition (g/kg dry yeast)	*Baker's yeast (aerobically grown)*	*Brewer's yeast (anaerobically grown)*
Carbohydrate	180-440	390-600
Protein	380-590	370-420
Ash	45-75	73-81
Nucleic acid (DNA and RNA)	52-95	39-43
Lipids	40-50	
P	10-19	14-20
S	3.9	
K	20-21	17
Na	0.12-0.3	0.7
Ca	0.6-0.75	1.3
Fe	0.02-0.03	0.1
Mg	1.3-1.65	2.3
Co	0.008	0.0002
Cu	0.008	0.033
Mn	0.0059	0.0057
Zn	0.170-0.197	0.0387
Cr	0.0005-0.0025	
Mn	0.008	
Ni	0.003	
Sn	0.003	
Mo	0.00004	
Li	0.000017	
V	0.00004	
Se	0.005	
Pantothenate (Coenzyme A)	0.065-0.10	0.110-0.120
Choline (membranes)	2.71-4.00	3.80-4.55
Thiamin (Vit B_1)	0.090 -0.165	0.092-0.150
Riboflavin (Vit B_2)	0.040-0.100	0.035-0.045
Nicotinic acid/niacin (NAD)	0.30-0.585	0.450
Pyridoxine (Vit B_6)	0.020-0.040	0.043-0.050
Biotin (biocytin)	0.0006-0.0013	0.001
p-aminobenzoate (folic)	0.160	0.005
Inositol (phospholipids)	3.0	3.9-5.0
Folic acid (1-C transfer)	0.013-0.015	0.010

Malony, 1998; Reed and Nagodawithana, 1991; Ingledew *et al.,* 1977; Patel and Ingledew, 1973; Peppler, 1970.

content to grow. Yeasts prefer to grow at acidic pH. Water is discussed in greater detail in the chapter by Ingledew in this volume.

A CARBON SOURCE (SUGARS AND STARCHES)

Low molecular weight sugars are the preferred carbohydrate source of *Saccharomyces*. Fermentable carbohydrates include the monosaccharides glucose, fructose, mannose and galactose as well as the disaccharides maltose and sucrose, and the trisaccharides, raffinose and maltotriose (uptake is strain dependent). Large polysaccharides such as starch and cellulose cannot be taken up by yeast in that form. Most brewing and distilling strains utilize sucrose, glucose, fructose, maltose and maltotriose in this approximate sequence, although some degree of overlap does occur. The initial step in the utilization of any sugar by yeast is either its hydrolysis outside the membrane (e.g. sucrose), followed by entry into the cell by some or all of the hydrolysis products or its intact passage across the cell membrane (e.g. maltose). Sucrose is the first sugar to disappear from the wort. The hydrolysis products, glucose and fructose, are then taken into the cell by facilitated diffusion (Figure 8). This is a passive uptake and both sugars are directly incorporated into the glycolytic pathway

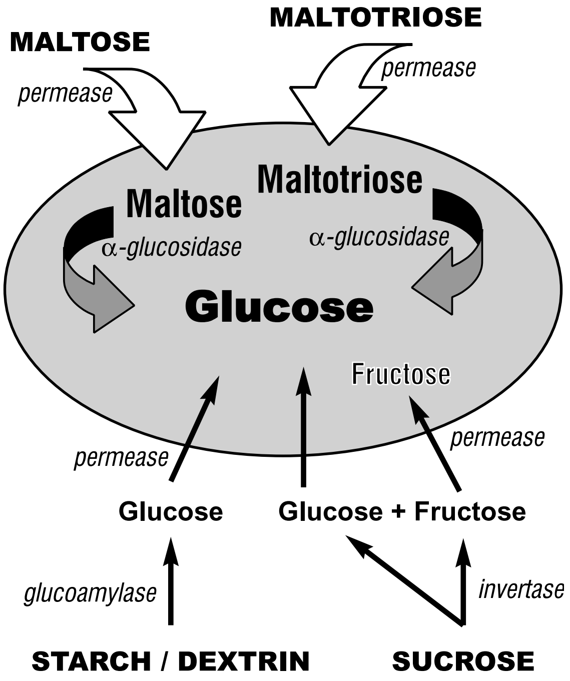


Figure 8. Uptake of sugars into the yeast cell.

immediately after entry into the cell (Figure 9). For the sugar sucrose (a disaccharide consisting of a glucose molecule and a fructose molecule) the enzyme invertase, which is located between the cell membrane and the cell wall, hydrolyses the sucrose to its two constituents, glucose and fructose, which then enter the cell by passive uptake. The hydrolysis of sucrose by the yeast's invertase is very rapid and so on a sugar profile the sucrose disappears very quickly from the substrate.

Maltose (two linked glucose molecules) and maltotriose (three linked glucose molecules) pass intact across the cell membrane by an active process, and once inside are hydrolyzed to glucose units by the α-glucosidase system. Maltotriose fermentation tends to begin later than that of maltose in a batch fermentation. Maltotetraose (four linked glucose molecules), higher polysaccharides (dextrins) and pentoses are not fermented by regular ethanol strains. If a very high glucose concentration relative to the other sugars is present, one can encounter 'hung' fermentations, due to a condition called glucose repression. Here the high concentrations of glucose prevent the yeast from synthesizing the enzymes needed to assimilate the other sugars present.

Figure 10 illustrates the typical sugar utilization pattern in a laboratory whisky mash fermentation where only the enzymes from the malt are available to break down the sugars and starches. A mash from a fuel ethanol fermentation would have commercial enzymes added to break the starch into smaller sugars that can be taken up by the yeast cell.

NITROGEN SOURCE

Yeast growth requires the uptake of nitrogen for the synthesis of protein and other nitrogenous components of the cell, but yeasts can only utilize low molecular weight nitrogenous materials such as inorganic ammonium ion, urea, amino acids and small peptides. The yeast cannot take up proteins or break down peptides larger than tripeptides. The extracellular proteolytic activity of yeast is negligible. Proteolysis does not occur in the fermentation, unless the yeast has autolysed. Nitrogen uptake slows or ceases later in the fermentation as yeast multiplication stops. Nitrogen is discussed in more detail later in this chapter.

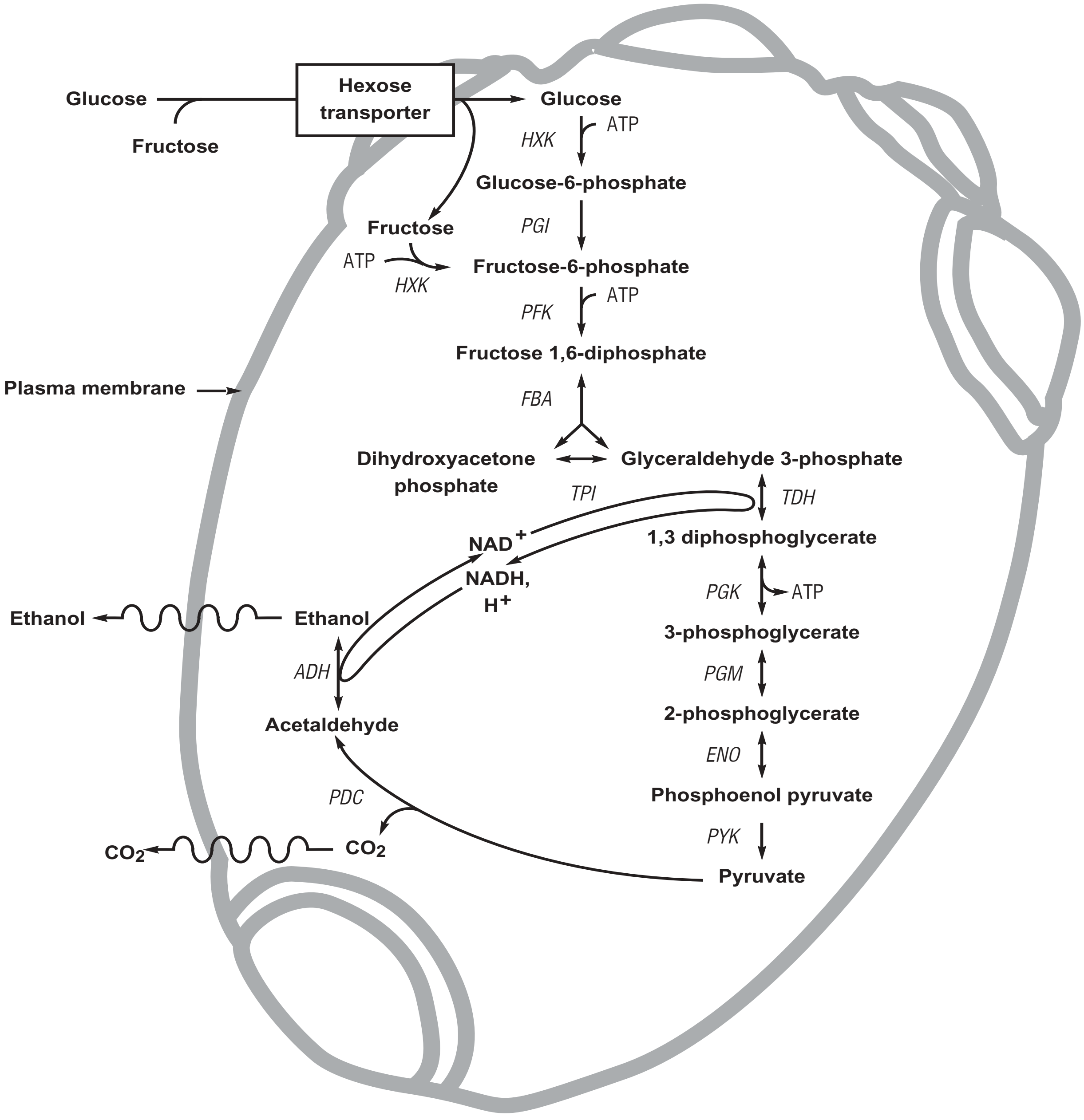

Glucose
Fructose
Hexose transporter
Glucose
HXK
ATP
Glucose-6-phosphate
PGI
Fructose
ATP
HXK
Fructose-6-phosphate
PFK
ATP
Fructose 1,6-diphosphate
Plasma membrane
FBA
Dihydroxyacetone phosphate
TPI
Glyceraldehyde 3-phosphate
TDH
NAD+
NADH, H+
1,3 diphosphoglycerate
PGK
ATP
Ethanol
Ethanol
ADH
3-phosphoglycerate
PGM
Acetaldehyde
2-phosphoglycerate
ENO
PDC
Phosphoenol pyruvate
PYK
CO2
CO2
Pyruvate

Figure 9. The glycolytic pathway (Embden-Meyerhof-Parnas) for sugar utilization.

Conversion of sugars to ethanol

Yeast is able to utilize sugars in the presence of oxygen (aerobically) and when oxygen is excluded (anaerobically). Aerobic breakdown (catabolism) produces more energy and is called *respiration*. Anaerobic catabolism produces less energy and is called *fermentation*.

Once sugars are inside the cell, they are converted via the glycolytic pathway (also called Embden-Myerhof-Parnas or EMP pathway) into pyruvate. The sequence of enzyme catalyzed reactions that oxidatively convert glucose to pyruvic acid in the yeast cytoplasm is known as *glycolysis* (Figure 9).

The very simplest expression for fermentation is the following equation:

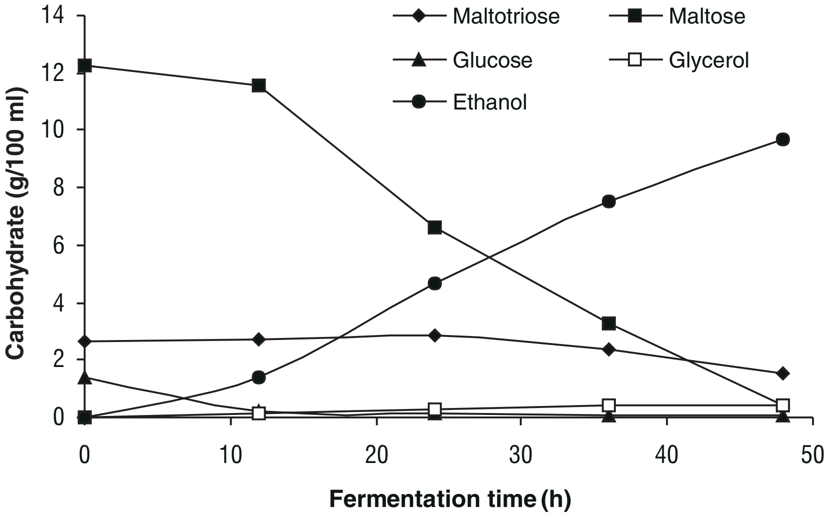


Figure 10. Uptake of sugars from a laboratory 19.3ºPlato/Brix whisky mash.

$$C_6H_{12}O_6 + 2\ P_i + 2\ ADP \longrightarrow 2\ C_2H_5OH + 2\ CO_2 + 2ATP + 2H_2O$$

Glucose ⟶ 2 carbon dioxide + 2 ethanol + energy

Mol wt 180 2 x 44 2 x 46

This equation, named after the French scientist Gay-Lussac, shows that glucose yields almost equal parts of carbon dioxide and ethanol as well as the production of energy. Part of the energy is used for cell metabolism and some of it will be lost as heat. What this equation does not address is that some of the sugar will be used for yeast growth and that there are other metabolites produced (glycerol, lactic and succinic acid, etc.). These other metabolites are small in quantity compared to the amount of ethanol produced.

The glycolytic (EMP) pathway operates in the presence or the absence of oxygen to convert glucose to pyruvic acid, energy and reduced nicotinamide adenine dinucleotide (NADH + H^+). When oxygen is abundant and the sugar level is kept low, little or no ethanol is made by the growing yeast. The yeast uses all of the sugar as energy for new yeast cell production (i.e. the baker's yeast propagation process). However when oxygen is reduced and/or the glucose levels exceed 0.1% (w/v), then ethanol is made. Under low oxygen conditions less than 5% of the sugar is metabolized for growth and the total cell yield is approximately 10% of what is possible under aeration and non-repressing glucose concentrations.

Not all glucose is metabolized by the glycolytic (EMP) pathway described above. Some of the sugar will be metabolized by a route called the hexose monophosphate pathway. This pathway also yields energy, but more importantly it yields pentose sugars, which are important in the synthesis of yeast nucleotides and yeast nucleic acids.

From glucose to pyruvate: glycolysis in yeast

Glucose is phosphorylated in two stages. Two ATPs are used to produce fructose 1,6 disphosphate, which is then split by the enzyme aldolase to form two 3-carbon triose phosphates. Inorganic phosphate is assimilated to form two triose diphosphates from which four H atoms are accepted by two molecules of oxidized NAD. Lastly, four ATPs are formed by transfer of phosphate from the triose diphosphates to ADP resulting in the formation of two molecules of pyruvic acid. Some of the pyruvic acid and other intermediates are used by the yeast cell through a variety of metabolic pathways as ‘building blocks’ for new yeast cells and some glycerol is made from the intermediate, dihydroxyacetone. However most of the pyruvic acid is immediately converted to ethanol and carbon dioxide (CO_2) (Figures 11 and 12).

Energy and the yeast cell

The yeast cell requires energy for three main activities.

i) Chemical energy: to synthesize complex biological molecules (i.e. make more yeast cell components)

ii) Transport: energy must be expended to transport nutrients in and out of the cell.

iii) Mechanical energy: cells need to move structures internally and this requires energy (i.e. budding).

There are two main carriers of energy in living cells, **A**denosine **T**ri**P**hosphate (ATP) and **N**icotinamide **A**denine **D**inucleotide (NAD^+). ATP is a carrier of chemical energy in the form of high energy phosphate bonds. NAD^+ is a carrier of hydrogen and electrons and is involved in many oxidation-reduction reactions in the cell. It can pick up and transport $2e^-$ and $2H^+$. The cell takes its energy source, converts it into NADH and ATP, and then uses these carriers to perform needed tasks in the cell. NAD^+ and ATP are not the only carriers of energy, but they are the major carriers.

Batch growth of yeast

The traditional concept of ethanol production was that growth occurred prior to the fermentation of most wort/mash sugars and that fermentation was carried out by non-growing stationary phase cells. It is now known that yeast growth, sugar utilization and ethanol production are coupled phenomena. The rate of fermentation by growing, exponential phase cells of an ale yeast strain is 33-fold higher than that of non-growing cells. Hence for maximum ethanol production, best practice is to keep the cells growing as long as possible at the highest possible cell concentration. When a yeast is transferred into a suitable fermentation medium under optimal growth conditions, the growth pattern illustrated in Figure 13 is seen.

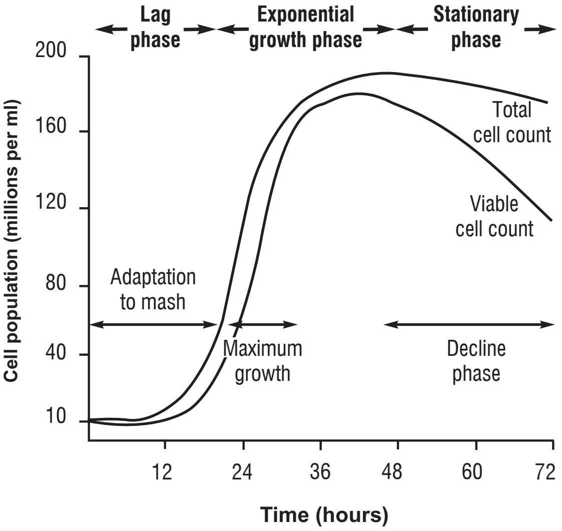


Figure 13. Typical batch growth curve for yeast.

CELL GROWTH PHASES

Lag phase. During this phase the yeast adapts to its new environment and activates its metabolism (i.e., synthesizing enzymes). It is a period of zero growth but a period of intense biochemical activity as the yeast cell adjusts from the previous culture conditions to the conditions in the fresh media. This phase ends with the first cell division.

Accelerating phase. Once cells transit from lag phase and commence active cell division they enter the acceleration phase before exponential growth. Here the rate of division continuously increases.

In a brewery, only an eight to 10-fold multiplication is possible, i.e. 3 cell generations, and possibly a fourth generation by a small proportion of cells. Further growth is not possible due to the lack of the essential membrane components (i.e. unsaturated fatty acids and sterols), which cannot be synthesized under the

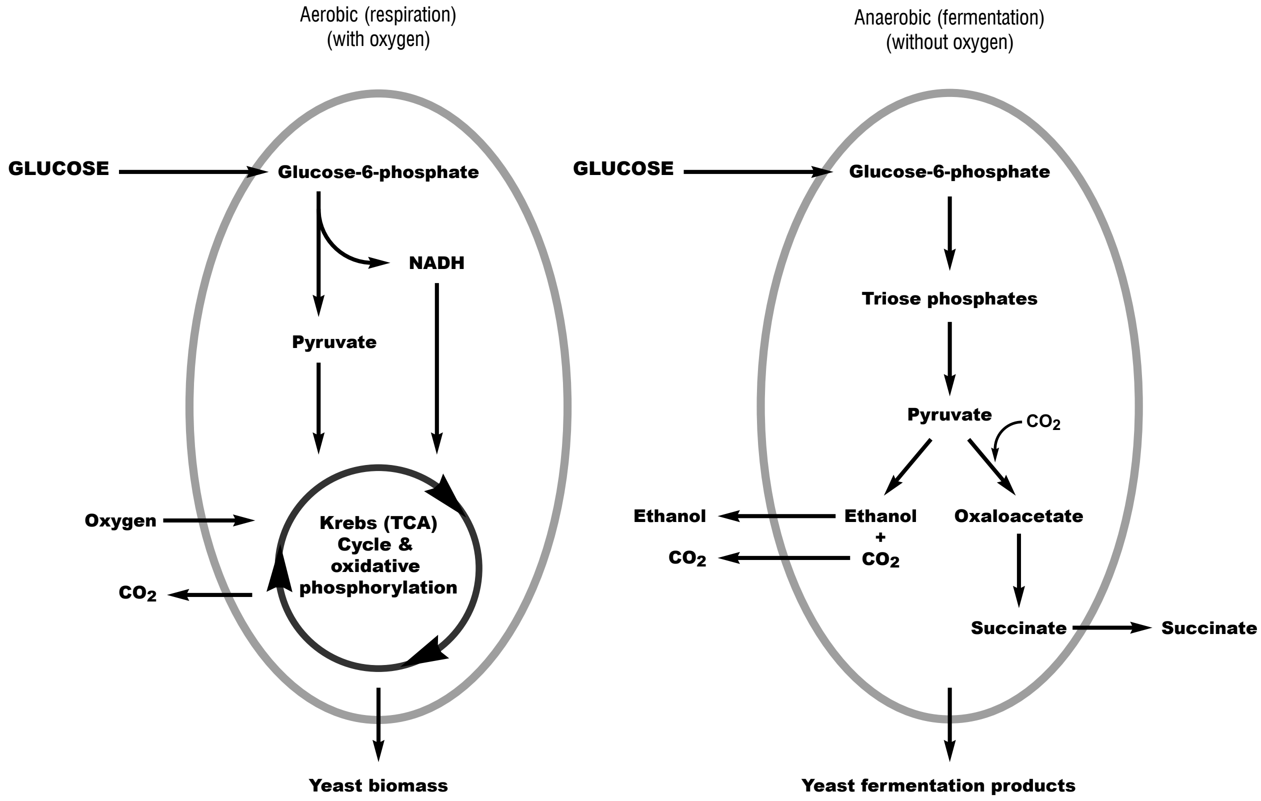

Aerobic (respiration)
(with oxygen)
Anaerobic (fermentation)
(without oxygen)
GLUCOSE
Glucose-6-phosphate
NADH
Pyruvate
Oxygen
CO2
Krebs (TCA) Cycle & oxidative phosphorylation
Yeast biomass
GLUCOSE
Glucose-6-phosphate
Triose phosphates
Pyruvate
CO2
Ethanol
Ethanol + CO2
Oxaloacetate
CO2
Succinate
Succinate
Yeast fermentation products

Figure 11. Aerobic and anaerobic catabolic pathways for sugar in yeast.

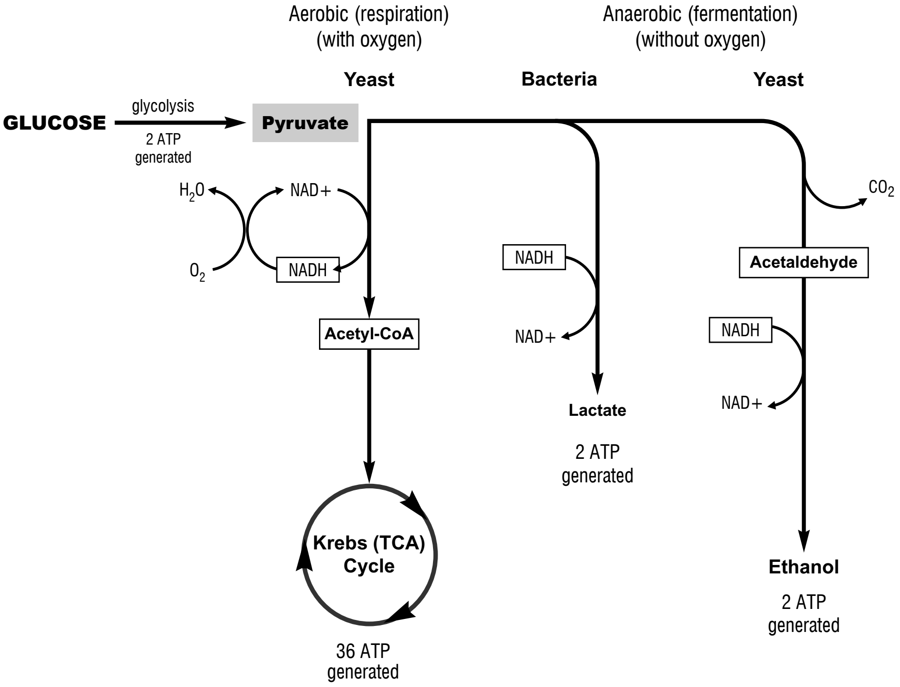

Aerobic (respiration)
(with oxygen)
Anaerobic (fermentation)
(without oxygen)
Yeast
Bacteria
Yeast
GLUCOSE
glycolysis
2 ATP generated
Pyruvate
H2O
O2
NAD+
NADH
Acetyl-CoA
Krebs (TCA) Cycle
36 ATP generated
NADH
NAD+
Lactate
2 ATP generated
CO2
Acetaldehyde
NADH
NAD+
Ethanol
2 ATP generated

Figure 12. Energy and the cell.

anaerobic conditions of fermentation (remembering that brewery yeast are recovered from previous anaerobic fermentations). Distillery yeast however, are pre-grown by the yeast manufacturer under vigorous aerobic conditions, generating an excess of the essential lipids, and when added to a well-aerated wort, a 20-fold multiplication is possible, i.e. slightly over four generations.

Log phase (exponential growth). In log phase growth rate is constant and maximal. It is a period of logarithmic cell doubling. The 'generation time' is the time during which the cell number doubles. Under optimal conditions generation time is between 90 and 120 minutes.

The yeast can be kept in this log phase by employing 'fed batch' culture conditions (i.e. incremental feeding of nutrients in step with yeast growth). This is used in the production of baker's yeast where the aim is to maximize cell mass and minimise ethanol production.

Deceleration phase. Growth rate decreases in this phase with the reduction in nutrients (i.e. carbohydrates) and/or the increase in growth inhibitors (i.e. ethanol).

Stationary phase. In the stationary phase the number of yeast cells stays constant (i.e., it is stationary with respect to the number of viable yeast cells). The number of new cells formed is balanced by the number that die. In the stationary phase, yeasts can survive for prolonged periods without added nutrients.

Declining phase. During this phase the death rate of the yeast cells exceeds the birth rate and the total cell number decreases.

Under well-controlled laboratory conditions the growth of yeast at constant temperature can be expressed as a straight line by plotting cell numbers logarithmically against actual time (hence the term log or exponential phase). It should be remembered that the growth curve shown is for a constant temperature. For plant fermentations, the large amounts of heat generated by yeast metabolism cause the temperature to rise throughout most of the log phase, and growth rate increases accordingly. This is an important difference from the textbook version of the microbial growth curve illustrated.

Propagation of yeast

Yeast propagated for the baking industry represents the largest amount of a single type of microbial biomass produced, with an annual production of baker's yeast netting $1.5 billion globally. The large-scale production of distilling and fuel ethanol yeasts follows the same principles as those employed for the manufacture of baker's yeast. A multi-stage propagation in a fermentation media usually consisting of sugar cane or beet molasses (as an inexpensive source of sucrose) with additional sources of nutrients added. The cell density progressively increases. The later stages are highly aerobic and the molasses medium is delivered slowly to avoid the Crabtree effect (i.e. suppression of respiration by high glucose levels where the cells continue to ferment despite the availability of oxygen) and to maximize respiratory growth. Yeast can be supplied to the ethanol industry as a slurry (yeast cream), compressed yeast or an active dry yeast.

Most of the yeast used in distilleries and fuel ethanol plants is purchased from manufacturers of specialty yeast appropriate for the purpose. Breweries in contrast tend to have their own proprietary strains and scale-up systems since they have the ability to crop and re-use their yeast.

Manufacturers propagate from proprietary laboratory stock cultures (best practice stores these cultures at ultra low temperatures) through a succession of fermentation vessels of increasing size. A culture medium of molasses (beet or cane) is supplemented with ammonium salts and any required trace nutrients. All cultures are grown with accurate temperature control at 30°C, with vigorous aeration (Campbell, 2003b). For efficient cultivation it is essential to maintain a low concentration of fermentable sugar in the culture medium. This avoids the Crabtree effect. The sugar is added as sterilized concentrated molasses, slowly at first and then increasing rapidly as the yeast biomass increases, so that there is a consistent level of about 0.5% sugar present. The yeast concentration will eventually surpass the ability of the aeration system of the culture vessel to maintain the necessary aerobic conditions for efficient growth. When the dissolved O_2 concentration approaches zero (after about 30 hrs growth) propagation is halted. The yeast culture can be harvested using a rotary vacuum filter. The circumference of the filter is a continuous strip of fabric coated with food-grade starch. As the drum rotates slowly through the culture in the trough, the spent culture medium is drawn under vacuum through the hollow spokes. The yeast is retained on the filter, scraped off and packaged.

YEAST SLURRY

Yeast in the slurry form is used primarily by brewers. They can recycle their yeast at a high viability (>90%). The slurry must be kept cool (4°C) and has a 3-7 day shelf life. If the yeast is fresh it can be acid-washed to lower the bacterial count. The slurry usually has a cell count in the range of ~3 x 10^9 cells/mL at 14-18% total solids.

COMPRESSED YEAST

Compressed yeast is more stable than slurry and requires cooling. The yeast is stored at 3–4ºC and has a 30 day shelf life. It loses viability at ~5-10 % a week. It can be rehydrated easily and has an indigenous bacterial count. The compressed yeast usually has a yeast count in the range of ~6-13 x 10^9 cells/mL at 33% total solids.

Compressed yeast is prepared at 4°C by adding salt to the yeast slurry and then passing the yeast through a cloth press or through a rotary drum vacuum filter. The resultant yeast has the consistency of butter and is then extruded into blocks, packaged, and both stored and shipped at 4°C. The yeast is sold as 25 kg bags of compressed moist yeast (24–33% dry weight) or in bulk as cream yeast slurry of about 18% dry weight. Before using compressed yeast it must first be slurried aseptically in a sterilized yeast mixing vessel to provide the required consistency for pitching. Cream yeast, which is delivered, stored and pitched in bulk, is often used for automated large-scale operations.

ACTIVE DRY YEAST (ADY)

Active dry yeast is used by most batch ethanol plants. It is also used to start continuous plants and to supplement fermentors. This yeast is dried under partial vacuum at 45–55ºC in an atmosphere of inert gas, usually nitrogen. Dried yeast (92–96% dry weight) has a long shelf life and does not require cold storage (but can be chilled for added security). It is very stable at room temperature and even more stable (over a year) if stored cool and under nitrogen gas. It loses ~7% viability/month at room temperature and <10% year at 4°C under nitrogen gas. The active dry yeast usually has a viable cell count in the range of ~2.2 x 10^{10} cells/g. It has an indigenous bacterial count. The process to make active dry yeast involves extruding the compressed yeast into spaghetti-like strands that are then dried in air lift dryers (fluidized bed). Reactivation (conditioning) of dried yeast at pitching is carried out to prevent loss of viability. It is important that rehydration is carried out under the prescribed conditions and once rehydrated the yeast must be used immediately.

The availability of a shelf-stable dried yeast inoculum where each gram contains ~2.2 x 10^{10} viable yeasts, is a major biotechnological advance. The yeast, which has been grown under extreme aeration for yield, is easier to store, handle and transport. Selected strains for individual processes are available (i.e. beverage versus fuel). The use of active dry yeast has reduced the need for yeast propagation expertise in the plants and increased the predictability of fermentations. Active dry yeasts are used in distilling and fuel alcohol plants to inoculate fermentors to recommended values of 1.0-2.0 million viable cells/mL per degree Plato/Brix of wort/mash.

CONDITIONING (ACTIVATING) THE YEAST

It is usually recommended that the active dry yeast is conditioned (according to the different manufacturer's instructions) to get optimal growth. (This is different from propagating a starter culture with the aim of growing up large quantities of yeast from a small lab culture.) For example, one manufacturer suggests a conditioning regime for active dry yeast for fuel ethanol plants where a yeast conditioning tank with a 16–18° Plato/Brix mash and a 1-2% glucose level is used. The recommended conditioning tank is 7–10% the size of the fermentor and the goal is 250-300 million cells/g. Temperature and nutrition at this stage are critical to ensure optimal yeast growth.

For distilled beverages, dried yeast can be added directly to the fermentation vessel or pre-mixed to a slurry with water. For direct inoculation, the wort cooler is adjusted to produce wort at 38ºC. The warm wort is collected to a depth of 10 cm in the washback and the required amount of dried yeast is sprinkled onto the wort, taking care to avoid clumping. After 5 min. the heat exchanger is adjusted to the normal set temperature, (~20ºC) and wort collection is completed.

For the pre-mixing method, sterile water or weak wort, 10 times the volume of dried yeast, is brought to 38ºC in a rehydration tank and yeast is added with stirring, to prevent clumping. After mixing for 5 min. the yeast slurry is pumped into the washback at normal set temperature. The pre-mixing method uses the same equipment as the preparation of a slurry from bags of pressed yeast, but for dried yeast the higher temperature of mixing is critical (Campbell, 2003b).

It should be noted that there are many different methods of conditioning the yeast and it will depend on the particular plant, the instructions with the particular type of yeast purchased and the expertise in the plant. The objectives of yeast propagation/conditioning are to deliver to a fermentor a large volume of yeast of high viability, high budding in the log phase of growth and with a very low level of infection.

There are many methods of propagating yeast (batch, continuous) all with pros and cons depending on the final use of the ethanol i.e. beverage or fuel, the unique characteristics of the plant, the technical skill in the plant, etc. The yeast propagator is the heart of a distillery; and it is important to remember the key objectives that are critical to the performance of a fermentation. Hence cost savings in this area are not advised since they are usually minimal when compared to the cost of production per gallon of ethanol. The loss of ethanol production that is possible when an aged yeast is used can be in the range of 1% alcohol, which can lead to a loss of $0.10 per gallon produced. Infection resident in a plant can cause a drop of 3% alcohol and a potential loss of $0.30 per gallon.

Wild yeast contamination

In the beverage alcohol industry wild yeast contamination is feared primarily due to the off flavors and odors that can be produced in addition to fermentation problems for the culture yeast. For non-beverage alcohol the concern is loss of alcohol due to non-utilization of the sugars for ethanol production. The production of excretion products such as acetic acid by wild yeast such as *Dekkera* (also called *Brettanomyces*) can inhibit the production yeast culture and cause serious fermentation problems in a plant. It is important to be familiar with the normal appearance of the production yeast under the microscope and to be constantly on the watch for deviations from the standard. Wild yeast will often (but not always) have a different appearance and exhibit very differently sized cells from the normal population. The cells can vary from elongated cells, to lemon shaped cells to oval cells. There may be buds with elongated necks or buds at odd angles. There are a number of specialized media that can be utilized to isolate and identify contaminating yeast cells (see Campbell, 2003a). In order to protect against wild yeast infections it is important to bring in a fresh culture yeast on a regular basis and ensure that there is a good cleaning and sanitation program in place. Once a wild yeast infection takes hold in a plant, that same contaminant often comes back numerous times and causes more infections. An extra-thorough cleaning and evaluation of problem spots in the plant is often necessary to rid the plant of the problem. Acid-washing of cropped yeast from a fermentation is ineffective against wild yeasts and many of the wild yeast are more acid-tolerant than the culture yeast. Washing will however reduce the number of bacteria present.

KILLER YEAST

Some strains of *Saccharomyces* secrete a proteinaceous toxin called a zymocide or killer toxin that is lethal to certain other strains of *Saccharomyces*. Toxin-producing strains are termed *killer yeasts* and susceptible strains are termed *sensitive yeasts*. There are strains that do not kill and are not themselves killed, and these are called *resistant*. The killer character of *Saccharomyces* is determined by the presence of two cytoplasmically located dsRNA plasmids (termed M and L). A wild yeast containing ‘killer factor' can have a negative impact in a beverage or fuel ethanol plant by annihilating the production strain and reducing the yield.

Temperature

Thermal damage to a yeast cell leads to a general denaturation of the yeast's proteins and nucleic acids. A yeast cell cannot regulate its temperature and all fermentation processes produce heat. The higher the temperature above the cell's optimal temperature for growth, the greater the loss of cell viability. Yeast thermotolerance is at its maximum when the external pH declines to 4.0. There is a general correlation between growth rate and stress sensitivity. Cells growing quickly in a glucose rich medium (the desired state for rapid ethanol production) are more sensitive to heat and other stresses than cells that are in stationary phase.

Many yeasts are capable of growth over a wide range from 5°C to 35°C. Commercial distilling yeast strains can ferment well at 32-35°C, but at higher temperatures metabolic activity rapidly declines. The optimal temperature for reproduction is usually lower and around 28°C. Yeasts purchased from ale breweries have a maximum growth temperature of 37.5-39.8°C while lager yeasts have a maximum growth temperature of 31.6-34.0°C. For example, if lager yeast is used as part of the inoculum in a whisky fermentation, they assist the distilling yeast only in the early part of the fermentation. Some *Saccharomyces* strains can survive 35-43°C under ideal conditions, but not under stressed conditions. It is important to remember that the growth optimum is species dependent as well as growth condition dependent. Figure 14 illustrates the effect of temperature on three different commercial yeast strains fermenting a 19.3° Plato/Brix whisky mash at three controlled temperatures. This figure illustrates the importance of selecting a strain that can function optimally at the temperature of the fermentation. All fermentation processes produce heat and the amount of cooling available and cost of cooling need to be taken into consideration when selecting a strain. Certain strains sold commercially have been constructed for their ability to withstand the stress of high ethanol and high temperature and are known to function well under these difficult conditions (Figure 15).

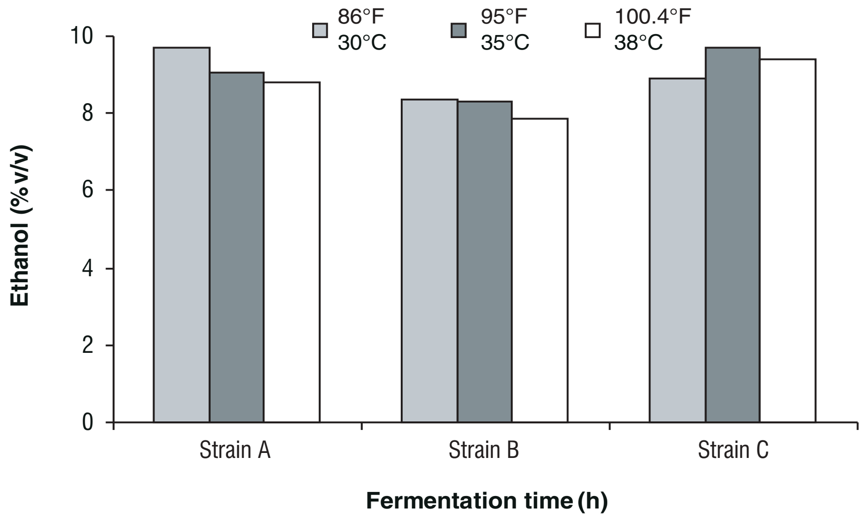


Figure 14. Effect of temperature on three different commercial yeast strains fermenting a 19.3° Plato/Brix whisky mash. Bars indicate final ethanol concentration at 48 hrs of fermentation. (Mash is 85% corn and 15% malted barley).

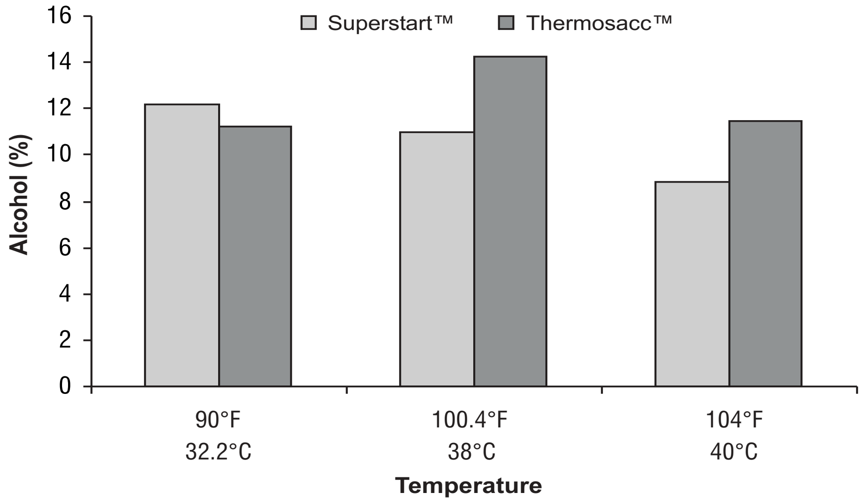


Figure 15. Ethanol production by two commercial yeast strains at three temperatures.

HEAT SHOCK AND COLD SHOCK

Yeast is sensitive to sudden temperature changes and shows signs of shock on sudden cooling with adverse effects on fermentation and cell multiplication. Cold shocking a yeast strain will inhibit budding and induce vacuolar rearrangements. Yeasts with elevated levels of trehalose are better able to survive cold storage. Yeast cells exposed to an elevated temperature will exhibit a molecular response called "the heat shock response" where certain proteins are synthesized which aid the yeast in responding to stress.

Pitching and viability

To follow the progress of a fermentation it is common practice to measure the quantity of yeast biomass or to count the number of cells. For pitching (inoculating) on a production scale, yeast is measured by mass: either directly as the number of 25 kg bags of yeast required, or indirectly as the volume of yeast slurry of standard concentration by weight. On a laboratory scale, counting the number of yeast cells per ml is usually employed. This requires a microscope and an engraved counting chamber slide called a haemocytometer (originally designed to count blood cells). Yeast is normally inoculated at a final concentration of ~1-2 lb active dry yeast/1000 US gallons (1 kg /4000 L) or 5 x 10^6 to 2 x 10^7 viable cells. A brewing rule of thumb is one million cells per degree Plato/Brix. So a 12° Plato/Brix wort or mash would be pitched at 12 x 10^6 cells and a 20° Plato/Brix wort/mash at 2 x 10^7 cells. Pitching the yeast at the correct level ensures reduced infection and improves the process efficiency thus reducing overall costs.

Viability involves the cell's ability to bud and grow, however slowly. Vitality is defined as a continuum of activity of the cell from very active to not active at all. Vitality tests are related to yeast growth, yeast physiological changes and fermentation performance.

Measurement of percentage viability alone is insufficient to guarantee the quality of the pitching yeast and hence the concept of yeast vitality (i.e. the ability of the yeast to ferment quickly and efficiently rather than simply being alive) is of interest. Various methods have been suggested as indicators of viability and vitality. Heggart *et al.* (1999) have extensively reviewed the factors that affect yeast vitality and viability.

In terms of measuring yeast health, to date no one test exists that is accurate, simple, inexpensive and rapid. It is often not possible to rely on a single method and a combination of methods is often employed. The evaluation of yeast viability includes methods based on cell replication (standard plate count and slide count technique), brightfield and fluorescent staining, measurement of intracellular ATP and NADH, and other methods such as electrokinetic potential and capacitance. Vitality assessment techniques include methods that measure metabolic activity (staining, microcalorimetry, vicinal diketone reduction, yeast protease activity, magnesium ion release, specific oxygen uptake rate, acidification power and intracellular pH), cellular components (ATP, adenylate energy charge, NADH, glycogen and trehalose, sterols and unsaturated fatty acids) and fermentative capacity (glycolytic flux rates, CO_2 measurement and short fermentation tests). A combination of various methods is often used to obtain an accurate picture of a yeast's fermentative capability.

One newer method that has been widely adopted in the brewing industry to assess yeast viability is capacitance measurement. When an appropriate radio frequency signal is applied to a yeast suspension, the dielectrical nature of the cell membrane permits a buildup of charge. Depending on the number of yeast present within the field, the resulting capacitance can be measured and is proportional to the radius and volume fraction of cells present. Since yeast cells are relatively constant in size, capacitance can be related to cell number and biomass. Dead cells with disrupted membranes do not generate a capacitance when a radio frequency is applied. Capacitance probes (Aber Instruments Ltd. Aberystwyth, UK) capable of providing an accurate measurement of viable yeast in real time have been successfully adopted by a number of breweries worldwide (Austin *et al.,* 1994).

Heggart *et al.,* (2000) have extensively reviewed the various techniques available for measuring yeast viability and vitality and the reader is directed to this review paper for further information on the many techniques available.

METHYLENE BLUE: THE MOST POPULAR VIABILITY TEST

Methylene blue has been in use since the 1920s and is recognized as a simple and standard method in the fermentation industry for measuring yeast viability. The buffered dye is added to a suspension of cells and examined under the microscope (ASBC, 1980). Methylene blue is taken up only weakly, if at all, by living cells, and any taken up is rapidly converted to the colorless reduced form by the metabolic activity of the cells. However, on death, the cells become readily permeable to methylene blue, and, since dead cells do not metabolize, the cells remain blue. There are a number of other redox dyes and variations on the makeup of the methylene blue dye buffers that have been suggested, but unfortunately these dyes are also unreliable with yeasts of lower viability than about 90% viable (Smart, 1999). Although the dye method has many shortcomings it does give a very fast evaluation of the status of a culture as long as one remembers that if more than 10% of the cells stain blue, the viability of the culture is in fact much lower. In other words the dye can be used to confidently confirm that a culture is very healthy (>90% viable) but is very inaccurate when a culture has a high number of dead cells in it.

To see if a culture is capable of reproducing (i.e. can the cell bud and eventually form a colony) a plate count can be carried out. When compared to a plate count, at viabilities above 90% the staining method consistently tends to overestimate cell viability by not more than 10–15%. When viability drops below 90–95% (as measured by a plate count), methylene blue counts can give values lower than 50%. At a plate viability of 0% (i.e. no growth on a plate), methylene blue may indicate apparent viabilities as high as 30–40% (Heggart *et al.,* 2000).

A well propagated yeast should be close to 100% viability, and should retain that value for several weeks if stored at 4ºC. Storage at ambient temperature is not recommended as it leads to a loss of viability. It also allows the rapid growth of bacteria from the inevitable initial low level of resident contamination. This low level of contamination can only be avoided if the yeast culture is propagated under pharmaceutical conditions. The extra expense for pharmaceutical conditions would make the product price prohibitive. Chilled yeast from an anaerobic fermentation does not have the storage stability of an aerobically grown yeast.

BUDDING INDEX

Cell counts that measure the number of budding yeast cells are often employed to determine if the yeasts are in the active desired growth phase since this is when ethanol is produced most rapidly. The yeasts are examined under the microscope with the use of a hemacytometer, and the number of budding cells counted to obtain a budding index. A budding index of ~30% is desirable as this indicates that the cells are in the rapid phase of growth.

pH

Yeast prefers an acid pH and its optimum pH is 5.0–5.2, but brewing and distilling strains are capable of good growth over the pH range of approximately 3.5 to 6.0.

During any fermentation, H^+ ions are excreted by the yeast, and this results in a pH decline in the media. For example, an all malt wort pitched with a pure culture of distiller's yeast will have an initial pH of ~5.2-5.5, which will fall to pH 4.0–4.5 (depending on the solids), and then increase slightly during the stationary phase due to release of amino acids from autolysing yeast cells. In fermentations showing late growth of lactic acid bacteria, these bacteria can use the yeast autolysate as a nutrient and further lower the pH to ~3.8. Contamination by lactic acid bacteria early in the fermentation is undesirable and reduced spirit yield results since the sugars used by the bacteria are then not available to the yeast for the production of ethanol. The loss of spirit yield in malt distilleries due to growth of *Lactobacillus* from the start of fermentation is reported as follows:

Up to 10^6 bacteria /ml	<1% loss
1–10 x 10^6 bacteria/ml	1–3% loss
1–10 x 10^7 bacteria/ml	3–5% loss
Above 10^8 bacteria/ml	>5% loss

No significant loss of spirit yield was observed when the lactic acid bacteria grew only at the end of the fermentation on the nutrients from the autolysed yeast (Dolan, 1976; Barbour and Priest, 1988). Changes in pH also affect the activity of α- and β-amylases and limit dextrinase, and consequently the hydrolysis of polysaccharides during the fermentation. The low pH associated with heavy early contamination by lactic acid bacteria reduces spirit yield if the enzymes cannot function well at that pH.

Yeast metabolism

Yeast produces a myriad of products. Table 2 summarizes some of the main products.

GLYCEROL

Glycerol is quantitatively the next most important product of yeast alcoholic fermentation after ethanol and carbon dioxide. It is an important compound in that it helps to maintain the cell's redox balance. When a yeast cell is growing, removal of pyruvate for cell biosynthesis can lead to a buildup of NADH, which can halt catabolism. To avoid this the cell reduces dihydroxyacetone phosphate to glycerol phosphate, which is then dephosphorylated to glycerol. The cell then excretes the glycerol into the growth medium (Figure 16). Redox balance and energy metabolism are highly integrated and the biosynthesis of new cellular material results in the production of a surplus of reducing equivalents of NADH. This is especially a problem under anaerobic conditions and *Saccharomyces* relies on glycerol production to

Table 2. Main products of yeast fermentation.

Alcohols	*Acids*	*Esters*	*Others*
Ethanol	Acetic	Ethyl acetate and any other	CO_2
Propanol	Caproic	combination of acids and	Acetaldehyde
Butanols	Caprylic	alcohols on the left	Diacetyl
Amyl alcohol	Lactic		H_2S
Glycerol	Pyruvic		
Phenethyl alcohol	Succinic		

(Campbell, 2003b)

re-oxidize the surplus of NADH formed during anaerobic conditions. However, during aerobic conditions, the surplus of reducing equivalents (as NADH) in the cytoplasm is delivered to the respiratory chain in the mitochondria.

The yeast must maintain a redox balance and glycerol carries out a redox balancing role, but it also plays a role as an osmoprotectant. The stress of high osmotic pressure and heat shock both enhance glycerol production. Glycerol accumulation inside the cell is very important for the survival of strains during osmotic stress. An increase in the fermentation temperature causes the yeast cell to produce larger quantities of glycerol. In a fuel ethanol fermentation, glycerol levels range from ~1.2–1.5%, again depending on a plethora of environmental factors.

In Germany in 1914, in order to produce large amounts of glycerol for nitroglycerine production, this yeast pathway was exploited. Using 'steered fermentation' acetaldehyde breakdown to ethanol was blocked by the addition of sodium bisulphite, and the glycolytic intermediates were reduced to give glycerol as the major fermentation product.

ACROLEIN

Acrolein has a very peppery, lacrimating and irritating effect on workers operating a still. Acrolein is produced from glycerol (Figure 17). It produces an intensely bitter taste when it combines with phenolic compounds. Certain contaminating lactic acid bacteria in a fermentation can degrade the glycerol present *via* a dehydratase enzyme to form 3-hydroxy-propionaldehyde, which under acidic conditions spontaneously degrades slowly to acrolein in storage. The process occurs more rapidly at higher temperatures.

SUCCINIC AND OTHER ORGANIC ACIDS

Organic acids, depending on the carbon chain length, can impart different flavors and aromas to beverages ranging from acidic to rancid to cheesy. Succinic acid is a main secondary end product of alcoholic fermentation. It is believed to be synthesized and secreted by yeasts either following limited operation of the citric acid (Kreb's or TCA) cycle or by reductive pathways involving some citric acid cycle enzymes. Low concentrations of pyruvic, malic, fumaric, oxaloacetic, citric, α-ketoglutaric, glutamic, propionic, lactic and acetic acids are also present as fermentation products. These are produced as intermediates of the citric acid (TCA) cycle (Figure 18).

The production of both glycerol and organic acids is greatly affected by the pH as illustrated in Table 3.

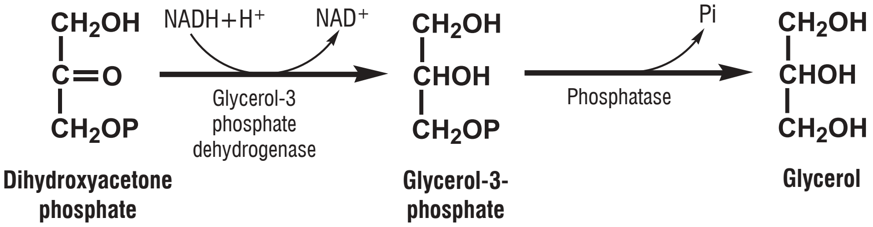


Figure 16. Conversion of dihydroxyacetone to glycerol phosphate to glycerol.

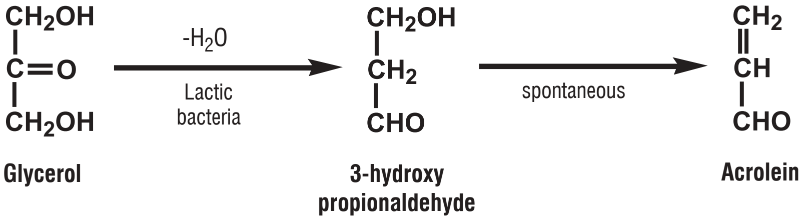


Figure 17. Degradation of glycerol to acrolein.

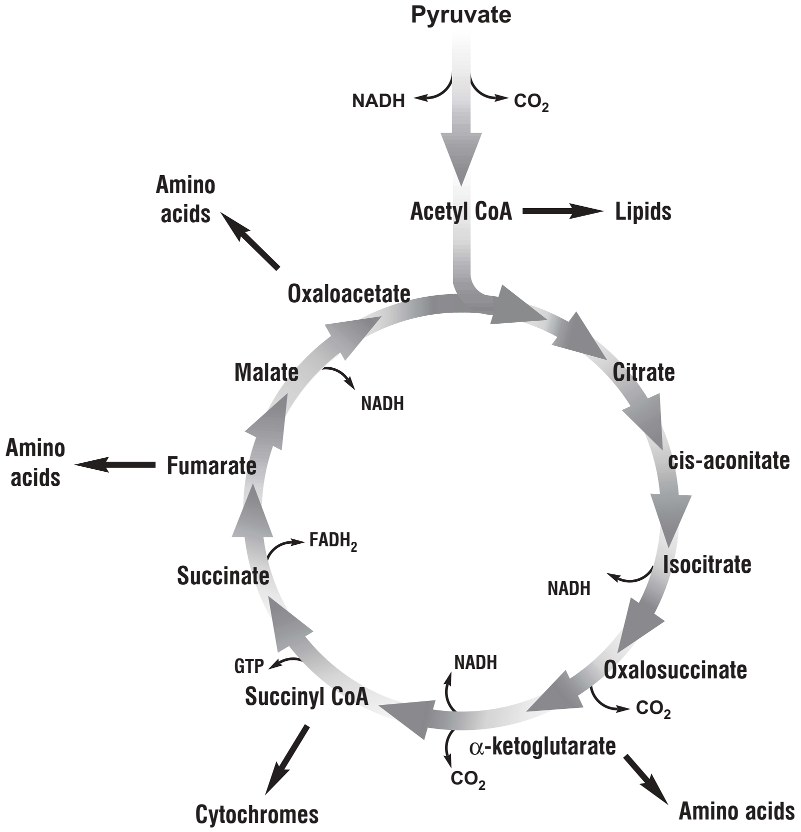

Pyruvate
NADH
CO_2
Acetyl CoA
Lipids
Amino acids
Oxaloacetate
Citrate
Malate
NADH
cis-aconitate
Amino acids
Fumarate
$FADH_2$
Succinate
Isocitrate
NADH
GTP
NADH
Oxalosuccinate
Succinyl CoA
CO_2
α-ketoglutarate
CO_2
Cytochromes
Amino acids

Figure 18. The citric acid cycle (also called Kreb's or TCA) for the synthesis of intermediates and energy under aerobic conditions.

Table 3. The influence of pH on production of glycerol and organic acids[1].

Product[a]	*pH 3.0*	*pH 4.0*	*pH 5.0*	*pH 6.0*	*pH 7.0*
Ethanol	171	177	173	161	150
Carbon dioxide	181	190	188	177	161
Glycerol	6.2	6.6	7.8	16.2	22.2
Acetic acid	0.5	0.7	0.8	4.0	8.7
Lactic acid	0.8	0.4	0.5	1.6	1.9

[1] Adapted from Neish and Blackwood, 1951 by permission.
[a] Millimoles of product per millimole glucose fermented.

SULFUR METABOLISM

The biosynthesis of S-containing amino acids and reduction of sulfate salts in the wort/mash are the key sulfur metabolism areas. In whisky distilling there are benefits from the remedial effects of copper in the stills, but with the normal ratio of copper surface area to pot still volume it is not possible (or desirable) to remove all sulfur compounds completely. Another source of sulfury off-flavors is the reduction of SO_4^{2-} to S^{2-} in the course of fermentation. Most of the resulting H_2S is stripped off by the evolution of CO_2 during fermentation. This can pose a problem in the collection of food-grade CO_2. High levels of H_2S and organic S compounds can also remain in the wash and affect quality of the distilled spirit if care is not taken.

OTHER COMPOUNDS

During fermentation the carbonyl compound found in highest concentration is acetaldehyde. It is formed during fermentation and is a metabolic branch point in the pathway leading from carbohydrate to ethanol. The acetaldehyde formed may either be reduced to ethanol, or oxidized to acetic acid, and in the final step of alcoholic fermentation, acetaldehyde is reduced to ethanol by an enzymatic reaction. The concentration of acetaldehyde varies during fermentation and reaches a maximum during the main fermentation and then decreases.

Diacetyl and pentane-2,3-dione both impart a characteristic aroma and taste described as 'buttery', 'honey- or toffee-like' or as 'butterscotch'. It is primarily a problem for brewers but in addition to being a yeast by-product, excessive levels can also be produced by certain strains of bacteria such as *Pediococcus* and *Lactobacillus*.

FLOCCULATION

Although brewers traditionally relied on the flocculence of the yeast to achieve separation of the yeast and beer at the end of fermentation (more common practice now is to separate by centrifugation), yeast for use in distilling is generally non-flocculent as flocculent yeast cells settle on heating surfaces of the still thereby restricting heat transfer. For beverage alcohol, if the yeast is charred, this will give rise to flavor defects in the spirit. The goal is to maintain a uniform yeast suspension throughout fermentation and distillation.

EFFECT OF CO_2 CONCENTRATION

Saccharomyces cerevisiae can tolerate CO_2 concentrations up to 2.23 volumes of CO_2 (v/v) in solution. Excessive CO_2 concentrations can inhibit total yeast growth, cause production of a range of volatiles and can affect the uptake of amino acids.

VITAMINS

Vitamins are important regulators and cofactors of numerous metabolic processes (Table 4). Their principal function is enzymatic and they generally act either as co-enzymes or precursors for fully active enzymes. Essential vitamin requirements for maximum fermentation rates are strain dependent. Yeast vary widely in their need for vitamins for their metabolism and, in a given strain, this need may also vary between active respiration and growth on the one hand, and alcoholic fermentation on the other. Almost all vitamins (except mesoinositol) are required by yeast to function as a part of a coenzyme, serving a catalytic function in yeast metabolism. Most strains have an absolute requirement for

Table 4. Vitamins and their functional role in yeast metabolism[1].

Vitamin	*Active form*	*Metabolic function(s)*	*Optimum concentration/ 100 ml [2]*
Biotin	Biotin	All carboxylation and decarboxylation reactions	0.56 µg
Calcium pantothenate	Coenzyme A	Keto acid oxidation reactions Fatty acid, amino acid, carbohydrate and choline metabolism. Acetylation reactions	45-65 µg
Thiamin (B_1)	Thiamin-pyrophosphate	Fermentative decarboxylation of pyruvate Rearrangements in pentose cycle, transketolase reactions, and isoleucine and valine biosynthesis	60 µg
Inositol	Inositol	Membrane phospholipids (structural)	9.3 mg (free) and 18.9 mg (total)
Niacin (B_4) (nicotinic acid)	NAD^+, $NADP^+$	Coenzymes in oxidation/reduction reactions (under anaerobiosis)	1000 - 1200 µg
Pyrdoxine (B_6), pyridoxal and pyridoxamine	Pyridoxal-phosphate	Amino acid metabolism, deamination, decarboxylation	85 µg
Riboflavin (B_2)	FMN, FAD	(Under anaerobiosis) as coenzymes in oxidation/reduction reactions	20 – 50 µg

[1] Adapted from Heggart *et al.* (1999).

[2] Optimal concentrations in 100 ml of a standard brewing wort.

biotin and many require pantothenate. A corn or grain mash should contain sufficient amounts of vitamins and they are not normally added directly to address a deficiency other than through the use of a yeast food. If deficiencies occur there can be resultant fermentation problems. When active dry yeast is used, since the yeast only multiplies a few generations, vitamin deficiencies are not usually encountered unless yeast recycling is practiced. Again, it must be stressed that each mash has unique characteristics that must be considered when yeast nutrition is an issue.

INORGANIC IONS

Yeast requires a number of inorganic ions for optimum growth and fermentation and this requirement varies with i) the yeast strain employed, ii) growth media differences, and iii) interactions with other constituents especially interactions among the trace metals. Important cations are zinc, manganese, magnesium, calcium, copper, potassium and iron (Table 5). With the exception of zinc, grain and corn based fermentation media are not usually deficient in these ions, but it is important to remember that differences in the fermentation substrate can cause large differences in metal content. A proper ratio between calcium and magnesium positively influences fermentation rates. An imbalance in inorganic nutrition is often reflected in complex and often subtle alterations of metabolic patterns and growth characteristics. The role played by many of these ionic species is both enzymatic and structural. The macro elements potassium, magnesium, calcium, iron, zinc, and manganese are required at concentrations between 0.1 and 1.0 mM. The micro elements cobalt, boron, cadmium, chromium, copper, iodine, molybdenum, nickel and vanadium are required between 0.1 and 100 mM. Minerals such as silver, arsenic, barium, mercury, lithium, nickel, osmium, lead, selenium and tellurium can be inhibitory when concentrations exceed 100 µM. Table 6 lists the effects of metal ions on yeast viability and vitality. For additional information on the effect of metal ions such as magnesium

Table 5. Metal ion requirements and function in the yeast cell[1].

Ion	*Function in yeast cells*	*Concentration*
Magnesium (Mg^{2+})	Stimulation of synthesis of essential fatty acids Alleviation of inhibitory heavy metal effects Regulation of cellular ionic levels Activation of membrane ATPase Maintain membrane integrity and permeability	2 – 4 mM
Potassium (K^+)	Central component in the regulation of divalent cation transport Essential for the uptake of $H_2PO_4^-$. Potent stimulator of glycolytic flux: significantly increases the levels of ATP, NAD(P)H, ADP and P_i	2 – 4 mM
Zinc (Zn^{2+})	Essential cofactor in a number of important metabolic enzymes, *e.g.* alcohol dehydrogenase Enhances riboflavin synthesis, activates acid and alkaline phosphatases Helps increase protein content of fermenting cells	4 – 8 µM
Calcium (Ca^{2+})	Not a requirement but may stimulate cell growth Protects membrane structure and helps maintain membrane permeability under adverse conditions Counteracts magnesium inhibition Plays an important role in flocculation	4.5 mM optimum for growth
Manganese (Mn^{2+})	Essential in trace amounts for yeast growth and metabolism Helps to regulate the effects of Zn^{2+} Stimulates the synthesis of proteins and biosynthesis of thiamin	2 – 4 µM
Copper (Cu^{2+})	Essential as an enzyme cofactor	1.5 µM
Iron (Fe^{2+})	Small amounts required for the function of haeme-enzymes	1 – 3 µM

[1] Adapted from Jones *et al.* (1981); Jones and Greenfield (1984); Heggart *et al.* (1999).

Table 6. Effects of metal ions on yeast viability and vitality[1].

Metal ion	*Effect on yeast*
Calcium (Ca^{2+})	Amino acid uptake inhibition above 1 mM and growth inhibition above 25 mM. Reduced rate of ethanol production and decrease in $Y_{x/s}$ and $Y_{p/s}$. Mg:Ca ratios that grossly favor Ca are detrimental to yeast growth and ethanol productivity through Ca antagonism of essential biochemical functions of Mg.
Copper (Cu^{2+})	Growth inhibition occurs at ~10 µM, with increasing potency up to 0.15 M (cell growth ceases). Causes changes in the yeast plasma membrane which can lead to the leakage of low molecular weight compounds and disturb the uptake of nutrients.
Iron (Fe^{2+})	Inhibitory only above 10–15 mM; excess Fe lowers activity of malate, pyruvate and succinate dehydrogenases.
Potassium (K^+)	Growth inhibition observed above 10 mM with total inhibition at about 2 M. Fermentation rate may be decreased above 4 – 10 mM.
Magnesium (Mg^{2+})	Total inhibition of growth reported at approximately 1 M.
Manganese (Mn^{2+})	Growth is progressively inhibited above 10 mM.
Sodium (Na^+)	No apparent metabolic role; at high concentrations, specific growth rate is decreased due to the diversion of energy to maintain an electrochemical gradient of Na^+ against passive diffusion or facilitated diffusion into the cell.
Zinc (Zn^{2+})	Deprivation in *S. cerevisiae* prevents budding and arrests cells in G_1 phase of cell cycle. Excess Zn added to wort inhibits fermentation and yeast growth. Inhibition of growth appears to occur above ~30 µM when Mn is below 7 µM; when Mn is above 7 µM, Zn can be as high as 1 mM before inhibition of growth occurs.

[1]Adapted from Heggart *et al.* (1999)

on ethanol tolerance in yeast, the reader is referred to the research work of Walker (1999).

Phosphorus

Phosphorus is essential to yeast cells for incorporation into structural molecules [e.g. phosphomannan and phospholipids, nucleic acids (DNA and RNA)] and phosphorylated metabolites (e.g. ATP and glucose-6-phosphate). Yeast stores polyphosphates as an energy reserve and uses this material during times of starvation. Phosphorus is commonly available in the form of inorganic orthophosphate ($H_2PO_4^-$) and this is rapidly metabolized to nucleoside triphosphate (ATP) on entry into the yeast cell. It can be added in the monobasic form or in the form of ammonium, sodium or potassium salts. A rule of thumb is that available phosphorus should be present at ~1-2% of the cell dry weight per unit volume of the medium.

Sulfur

Inorganic sulfur, in the form of sulfate anions, is transported *via* an active process by yeast into the cell for assimilation into sulfur containing amino acids such as methionine and tripeptides such as glutathione (glutamic acid-cysteine-glycine). In the presence of excess sulfate, yeast can store sulfur intracellularly in the form of glutathione, which can account for as much as 1% of the cellular dry weight. Under conditions of sulfate limitation or starvation, the glutathione in the yeast can act as a sulfur source for the biosynthesis of amino acids. Sulfur is required at ~0.3–0.5% of the weight of expected yeast mass per volume of medium.

Magnesium

Magnesium is the most abundant intracellular divalent cation in yeast and it acts primarily as an enzyme cofactor. It exerts a protective effect on yeast cultures subjected to stress conditions such as temperature and osmotic pressure, as well as playing a role in alcohol tolerance. Magnesium stimulates fermentation during the metabolism of high gravity worts/mashes. The requirement for magnesium is small and is usually amply provided by the raw materials and water. Exogenous magnesium exerts a protective effect on yeast cells subjected to physical and chemical stresses (Walker, 1999).

Zinc

Trace levels of Zn^{2+} are essential for yeast growth and other metal ions cannot fill this requirement. Zinc is a cofactor in a number of enzymatic reactions within the cell and the important fermentation enzyme, alcohol dehydrogenase, is a zinc metalloenzyme. Certain yeast strains may require more zinc than occurs naturally in wort/mash, and supplementation with a yeast food is used. Zinc deficiency results in low yields of yeast (can inhibit budding) and slow fermentations. A concentration of 0.3 ppm Zn in the medium is considered adequate and higher concentrations of zinc (1-5 ppm) may inhibit yeast activity, but this depends, at least in part, on the level of manganese present.

Potassium

Potassium is the most prevalent cation in the yeast cytoplasm (~150 mM). Uptake of K^+ in yeast requires an active transport mechanism and the presence of glucose or a fermentable or respirable substrate. Transport of K^+ is associated with the transfer of H^+ ions from within the cell.

Sodium

Yeast cells do not accumulate Na^+ ions under normal growth conditions and continuously excrete Na^+ in order to maintain very low cytoplasmic concentrations of this cation. In the presence of high salt concentrations, the yeast cells osmoregulate by producing intracellular compatible solutes such as glycerol and arabitol.

Manganese

Manganese (Mn) is essential for yeast growth and metabolism and in trace levels acts as an intracellular regulator of key enzymes. Manganese ions have been reported to stimulate fermentation.

Calcium

Calcium (Ca) stimulates yeast growth but it is not a growth requirement. It is involved in membrane structure and function. Yeast cells maintain cytosolic Ca^{2+} at very low levels. The significance of Ca^{2+} uptake in yeast lies in the multifunctional role of this cation as a modulator of growth and metabolic responses. Calcium also plays an important role in flocculation.

Copper and iron

Copper (Cu) and iron (Fe) ions are essential nutrients for yeast and act as cofactors in several enzymes including the redox pigments of the respiratory chain. Copper is an essential micronutrient at low concentrations, but is toxic at higher concentrations. Strains of yeast differ in their sensitivity to copper and negative effects on fermentation can be seen starting at concentrations of >10 ppm. Iron is usually abundantly provided for in brewer's wort where the average content is 0.1 ppm.

HIGHER ALCOHOL PRODUCTION

From a quantitative point of view, the most important higher alcohols (fusel oils) produced are n-propanol, amyl alcohol, isoamyl alcohol and phenethyl alcohol. In addition, over 40 other alcohols have been identified. Regulation of the biosynthesis of higher alcohols is complex since they may be produced as by-products of amino acid catabolism or *via* pyruvate derived from carbohydrate metabolism (Figure 19).

The catabolic route (i.e. Ehrlich pathway) (catabolic defined as a biochemical process in which organic compounds are digested, usually an energy-liberating process) involves a pathway in which the keto acid produced from an amino acid transamination is decarboxylated to the corresponding aldehyde, then reduced to the alcohol *via* an NAD-linked dehydrogenase (Figure 20). In this way, for example, isobutanol may be produced from valine, 3-methyl-1-butanol from leucine and 2-methyl-1-butanol from isoleucine. They are by-products of the amino acid assimilation pathway.

The anabolic route of *de novo* synthesis (i.e. biosynthesis pathway) (anabolic defined as a biochemical process involving the synthesis of organic compounds, usually an energy-utilizing process) utilizes the same pathways as those involved in the biosynthesis of amino acids. As in the catabolic route, the keto acid intermediate is decarboxylated and the resultant aldehyde reduced to the alcohol.

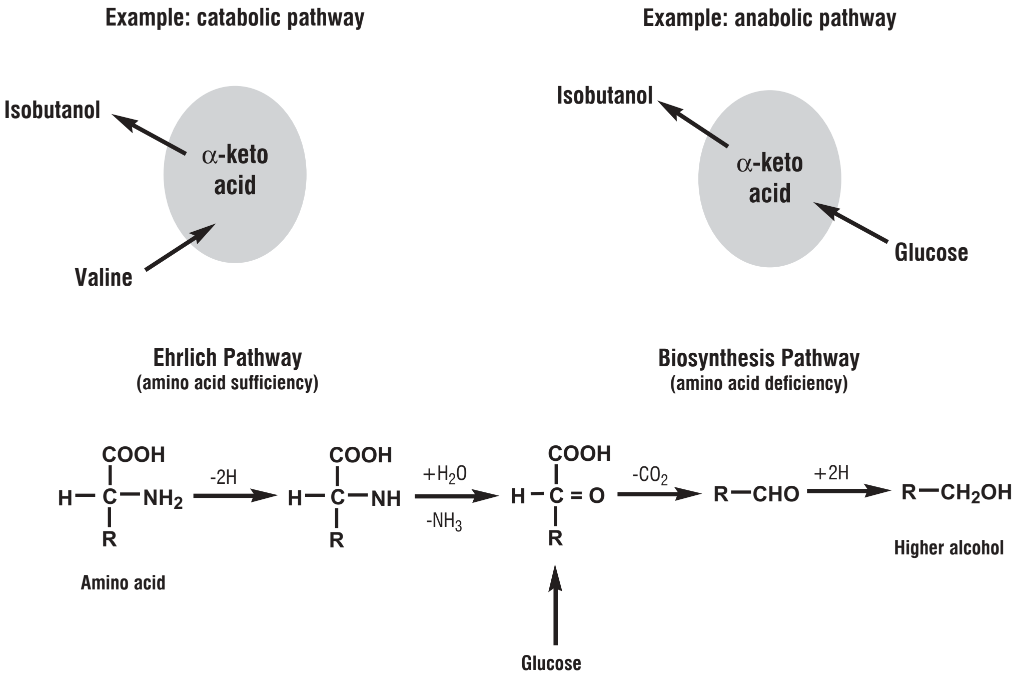

Example: catabolic pathway
Example: anabolic pathway
Isobutanol
α-keto acid
Valine
Isobutanol
α-keto acid
Glucose
Ehrlich Pathway
(amino acid sufficiency)
Biosynthesis Pathway
(amino acid deficiency)
COOH
H—C—NH2
R
Amino acid
-2H
H—C—NH
+H2O
-NH3
H—C=O
-CO2
R—CHO
+2H
R—CH2OH
Higher alcohol
Glucose

Figure 19. Higher alcohols (fusel oils) are produced by yeast metabolism *via* two routes both of which utilize α-keto acid (adapted from Walker, 1999).

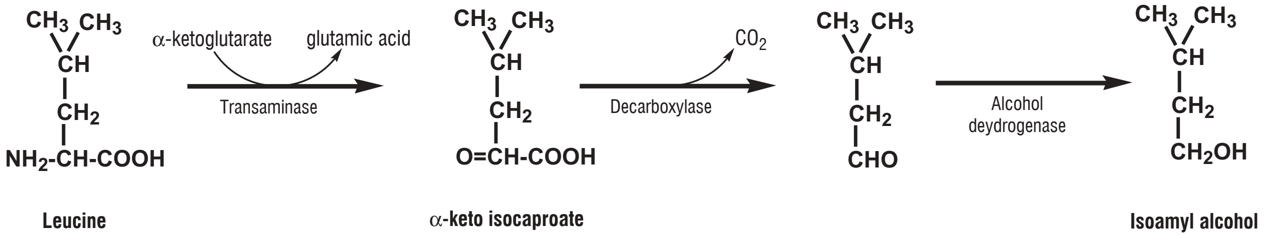

CH3 CH3
CH
CH2
NH2-CH-COOH
Leucine
α-ketoglutarate
glutamic acid
Transaminase
O=CH-COOH
α-keto isocaproate
CO2
Decarboxylase
CHO
Alcohol deydrogenase
CH2OH
Isoamyl alcohol

Figure 20. Higher alcohol production. Conversion of the amino acid leucine to isoamyl alcohol.

The relative contribution made by the two routes varies with individual higher alcohols. Since there is no corresponding amino acid, the anabolic route appears to be the sole mechanism for the formation of n-propanol.

The concentration of amino acids in the medium is important. If there are only low levels of assimilable nitrogen then the anabolic route is predominant. High concentrations of wort amino nitrogen favor the catabolic pathway due to feedback inhibition of the amino acid synthetic pathways by their respective products.

Both the level and the composition of the yeast-assimilable nitrogen in the mash influence the formation of higher alcohols. Low levels of usable nitrogen result in less yeast growth and increased yields of higher alcohols. When assimilable free amino nitrogen is increased in the mash, the anabolic (biosynthesis) pathway becomes less dominant and the Ehrlich pathway takes over. If ammonium ion or urea is the main nitrogen source, higher alcohols are made via the anabolic (synthetic) pathway.

ESTERS

Esters are a product of yeast metabolism and there are over 100 distinct esters identified in fermentation beverages. The most abundant ester is ethyl acetate (fruity/solvent). Other esters produced include isoamyl acetate (banana/apple), isobutyl acetate (banana/fruity), ethyl caproate (apple/aniseed) and 2-phenylethylacetate. Esters are minor components with ethyl acetate being present at over 10 mg/L and the others typically less than 1 mg/L.

Biosynthesis of the esters involves two enzymes, acyl-CoA synthetase and alcohol acetyl transferase. There appears to be more than one alcohol acetyl transferase. The metabolic role for ester formation is still not well understood but it may provide a route for reducing the toxic effects of fatty acids by their esterification and removal.

Fermentation components that promote yeast growth tend to decrease ester levels; and increased oxygenation tends to reduce ester levels. Higher temperatures lead to increased ester formation and increased levels of higher alcohols.

ORGANIC ACIDS FROM CONTAMINATING BACTERIA

Contaminating bacteria can alter the normal profile of organic acids. Lactobacilli are very ethanol tolerant and are capable of very rapid growth in distillery mashes. Increases in lactic and acetic acids are observed when there is contamination and these acids can inhibit the growth of yeast if produced in excess, or if recycled and allowed to build up in backset. Concentrations of lactic acid over 0.8% (w/v) and of acetic acid over 0.05% (w/v) affect the growth of yeast.

In addition to affecting the growth of yeast, the low pH can affect continuing glucoamylase activity on residual dextrins in the fermentor. Depending on the nitrogen source in the mash, levels of acid can vary from 0.5-1.4 g/L and can quickly rise to over 15 g/L when there is heavy bacterial or wild yeast contamination.

Infection always means loss of ethanol. For every molecule of lactic acid produced there is a loss of one molecule of ethanol. Thus for every 90 g of lactic acid made, 46 g of ethanol could have been made. Likewise, for acetic acid where for every 60 g of acetic acid made, 46 g of ethanol could have been made.

OXYGEN AND YEAST GROWTH

Saccharomyces cannot grow indefinitely under anaerobic conditions unless it is supplied with certain nutrient supplements, specifically sterols and unsaturated fatty acids. These two compounds are essential components of the yeast cell membrane and yeast cannot synthesize them anaerobically. As the yeast grows and buds, it shares its initial fatty acids and sterols with the daughter cell. This sharing, as the yeast cell continues to grow and bud, eventually 'dilutes out' the required fatty acids and sterols in the cell membrane. After an extended number of generations without a source of oxygen or pre-formed lipid precursors of the necessary cell membrane constituents, the yeast will be unable to ferment sugars. This is not a problem under aerobic conditions since the required sterols and unsaturated fatty acids are synthesized *de novo* from carbohydrates. For the fermentation to proceed rapidly it is critical that there is adequate yeast growth.

There is a wide range of oxygen requirements among yeast strains and several authors have suggested that the strains be divided into four classes in regard to their oxygen requirement for fermentation, with Group 1 yeast being fairly insensitive to low oxygen content and Group 4 yeast having a high oxygen requirement. The range varies from 4 ppm dissolved oxygen to over 40 ppm dissolved oxygen.

It is important to remember that the amount of air or oxygen injected into the fermentation medium may bear little relationship to the amount of oxygen that actually dissolves. Thus it is necessary to measure the oxygen in solution in order to know what is available to the yeast. The amount of oxygen required for sufficient sterol synthesis, yeast growth and satisfactory fermentation differs widely among different strains of yeast. There is a fine balance that must be achieved. If the oxygen concentration is too low then the lack of sterol synthesis can restrict yeast growth resulting in fermentation problems. If the concentration of oxygen is excessive, then although there is a rapid fermentation, there is also high yeast growth with a consequent reduction in ethanol yield since the substrate has been used for yeast growth rather than ethanol production.

A yeast with a high oxygen requirement, one approaching air saturation in a 10° Plato/Brix brewing wort (specific gravity 1.040), will be unable to ferment an air-saturated high gravity wort since not enough oxygen is available. The concentration of oxygen at 100% air saturation is inversely proportional to the specific gravity (and the temperature), thus an air saturated wort of 10° Plato/Brix would contain 8.5 ppm O_2, whereas a wort of 17° Plato/Brix (specific gravity 1.070) would only contain 7.9 ppm O_2. Oxygen solubility is also lower in mashes poised at >35°C that contain increased amounts of solids.

Mash is fermented under anaerobic conditions and the very small amount of air (8-20 ppm O_2) that should be supplied around the time of initial inoculation can be added by 'rousing' the cooled mash using air or bottled oxygen, or by 'splash filling' the fermentor just prior to adding the yeast. This small amount of oxygen, *if* used aerobically, would only be enough to allow respiration of 0.008% (w/v) glucose so there is no large negative effect on sugar utilization. Since there is excessive glucose present in the mash, the oxygen is probably used anaerobically. Oxygenating at low levels at the initiation of fermentation has been carried out for many years by the brewing industry and the most effective time to ensure oxygen availability is when the yeast begins to actively grow. The amount of oxygen is important, but the timing of the supply is also key. The oxygen must be supplied during the growth and division phase of the yeast. In distillery practice, it may be sufficient to splash fill tankage just prior to fermentation, especially if active dry (aerobically grown) yeasts are used.

Clear substrates without insoluble lipid materials present may require more dissolved oxygen. For example, with high gravity brewer's worts that have been filtered and are almost devoid of insolubles, bottled oxygen is used to oxygenate. The amount of oxygen needed by yeast growing anaerobically is small, but important, and deprivation leads to fermentation difficulties.

Reproductive growth eventually ceases when the limiting value of sterols is reached. When cell multiplication stops, the rapid uptake of nitrogen and carbohydrates is affected and cells have a reduced ability to complete the fermentation. In addition they are more susceptible to external stresses such as temperature and pH. This has an impact on the rate of ethanol production. The limiting level of unsaturated fatty acids for brewing yeast is ~0.5%.

Active dry yeasts (ADY), which are grown under conditions of high aeration, have a much higher unsaturated fatty acid and sterol content than yeasts propagated or conditioned 'in house' in an ethanol fermentation plant. This high 'credit' of sterols and unsaturated fatty acids allows the active dry yeasts to complete a fermentation without the same oxygen (or sterol/fatty acid) requirement in the fermentation broth. It is important to note that these yeasts are never reused. Distillery practices do not allow for harvesting.

Many distilleries and fuel alcohol plants use active dry yeasts. Aeration through 'splash filling' of batch fermenters or circulation of mash through heat exchangers for cooling purposes is probably adequate if active dry yeast is used. Aeration at the heat exchanger should be done on the cold side of the exchanger to ensure maximum solubility of oxygen on injection (remembering that oxygen is more soluble at lower temperatures).

Yeast sterols and oxygen

Sterols in aerobically-grown yeast may reach 1% total dry weight, but with anaerobic growth and no usable sterols in the medium, they dilute out to a value of less than 0.1%. There are several sterols found in yeast of which ergosterol is the most prominent. Sterols play a key role in regulating membrane fluidity. When insufficient oxygen is available for membrane synthesis, yeast cells fail to grow and loss of membrane integrity results in cell death. The regulation of sterol synthesis is extremely complex. Yeast cells accumulate sterols into the membrane until they have met bulk requirement needs. After this, additional sterols are esterified to long chain fatty acids and stored in intracellular lipid vesicles. The most abundant sterol of the sterol pool is zymosterol. In an aerobic culture these sterol esters accumulate during the stationary phase of growth and when these cells are re-inoculated into fresh medium these steryl esters are then hydrolyzed and incorporated into the growing cell membranes.

Medium length fatty acids (C6–C10) arise via the activity of fatty acid synthetase as intermediates in the formation of longer chain length fatty acids, which are incorporated into the various classes of yeast lipids. The fatty acid biosynthetic pathways lead to the saturated and unsaturated fatty acids used in membrane lipids. Unless oxygen is present in small quantities when growth begins, the desaturation steps do not proceed and unsaturated fatty acids and sterols cannot be synthesized by the yeast cell.

GLYCOGEN

Glycogen is the yeast's major reserve carbohydrate and it is similar in formation and structure to plant amylopectin. It is a polymer of α–D-glucose with a molecular weight of ~10^8. It has a branched structure containing chains of 10–14 residues of α–D-glucose joined by 1→4 linkages. Individual chains are connected by (1→6) α–D-glucosidic linkages (Figure 21).

Glycogen serves as a store of biochemical energy during the lag phase of fermentation when the energy demand is intense for the synthesis of such compounds as sterols and fatty acids. This intracellular source of glucose fuels lipid synthesis at the same time as oxygen is available to the cell. Dissimilation of glycogen and the synthesis of lipid are both rapid. The hydrolysis of glycogen from approximately 20-30% to 5% and the corresponding production of lipid from 5% to 11.5% of the cell dry weight occurs within the first few hours after pitching. Toward the later stages of fermentation, the yeast restores its reserve of glycogen.

TREHALOSE

Trehalose is a non-reducing disaccharide consisting of two glucose units linked together by an α-1, 1-glycosidic bond (Figure 22). Trehalose is present in particularly high concentrations in resting and stressed yeast cells. During sporulation trehalose is virtually the only sugar present in the yeast spores. Trehalose plays a protective role in osmoregulation. It protects cells during conditions of nutrient depletion, against toxic chemicals (ethanol, oxygen radicals, heavy metals) and starvation. It also plays a role in improving cell resistance to high and low temperatures as well as resistance to dessication. This protective role may be due to the stabilising effect of trehalose on cell membranes. It is believed that the induction of heat shock proteins and the accumulation of trehalose are related. Trehalose accumulation occurs during late stationary phase or in response to environmental changes. Trehalose levels are important in the preparation of active dried yeast since trehalose acts as a protectant during the drying phase.

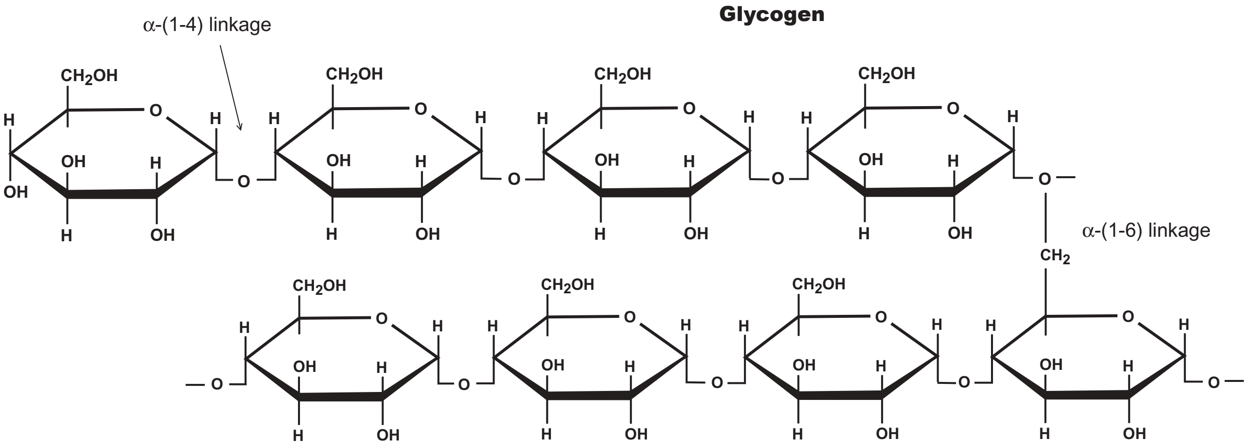


Figure 21. Structure of glycogen: a high molecular weight polymer with branched-chain structure composed of D-glucopyranose residues.

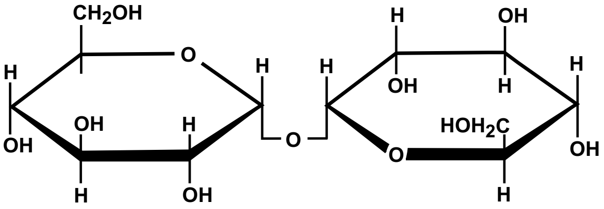


Figure 22. Structure of trehalose ($C_{12}H_{22}O_{11}$). An α-D-glucopyranosyl- α-D-glucopyranosyl.

YEAST MUTATIONS

Yeast mutations are a common occurrence throughout growth and fermentation, but they are usually recessive mutations, due to functional loss of a single gene. Since industrial strains are usually aneuploid or polyploid, the strains will be phenotypically normal and only if the mutation takes place in both complementary genes will the recessive character be expressed.

If the mutation weakens the yeast, the mutated strain will be unable to compete and soon be outgrown by the non-mutated yeast population. The accepted view was that industrial yeast strains were genetically very stable, however, with techniques such as DNA fingerprinting it has been found that instability is not uncommon in many production brewer's yeast strains. This finding suggests that great care must be taken in regard to yeast generation production specifications.

Some commonly observed characteristics resulting from yeast mutation that can negatively affect fermentation are: i) The tendency of yeast strains to mutate from flocculent to non-flocculent ii) The loss of ability to ferment maltotriose and iii) the presence of respiratory deficient mutants.

Serial repitching exposes the yeast to a number of stresses that lead to genetic drift and mutation. The most common mutation encountered is the respiratory deficient (RD) or 'petite mutation' arising as a result of the accumulation of sublethal DNA damage. The mutation arises spontaneously when a segment of the DNA in the mitochondrion becomes defective to form a flawed mitochondrial genome. The mitochondria are then unable to synthesize certain proteins.

This type of mutation is also called the 'petite' mutation because colonies with such a mutation are usually much smaller than the normal respiratory sufficient culture. The respiratory deficient mutation occurs at frequencies of between 0.5% and 5% of the population but in some strains much higher figures have been reported. Deficiencies in mitochondrial function result in a diminished ability to function aerobically (hence the name respiratory deficient), and as a result these yeasts are unable to metabolize the non-fermentable carbon sources lactate, glycerol or ethanol.

Flocculation, cell wall and plasma membrane structure, and cellular morphology are all affected by this mutation. As a result the following effects have been observed: slower fermentation rates, higher dead cell counts, reduced biomass production and flocculation ability, alterations in sugar uptake and metabolic by-product formation, and reduced tolerance to stress factors such as ethanol and temperature.

Nitrogen and yeast growth

Stuck or sluggish fermentations occur when the rate of sugar utilization becomes very slow or protracted, especially toward the end of a fermentation. When the sugar content is high and vital nutrients are lacking, a stuck fermentation can occur. An inadequate level of assimilable nitrogen is often the culprit.

How much nitrogen does a yeast cell require to grow and in what form? In traditional batch fermentations there is a 3 to 10-fold increase in yeast cell number. Yeast is normally inoculated to a final concentration of 5 x 10^6 to 2 x 10^7 viable cells per mL. In terms of pitching weight, this is approximately 2 lb of active dry yeast per 1000 US gallons (or 1 kg/4000 L). After growth in the fermentation medium the yeast produced weighs from 6 to 20 lb in 1000 gallons (or 3–10 kg/4000L).

The mash is a microbiological growth medium and if the right nutrients are not supplied in the mash then the yeast will not grow well (or ferment rapidly) and lower levels of ethanol will result. Table 7 illustrates the total and useable levels of free amino nitrogen (FAN) from a number of sources.

Table 7. FAN levels of typical mashes (mg/L mash*).

	Total FAN	*Useable FAN*
Wheat	82	64
Barley	84	62
Hulless barley	124	100
Oats	193	159
Hulless oats	184	130
Rye	103	83
Molasses	267	141
Corn	70	58
Starch slurry	~0	~0

*all mashes normalized to 22% solids

Table 8 illustrates the FAN levels in a mash with backset from a corn dry milling plant and Table 9 is a corn wet milling example illustrating FAN levels with backset and steep water.

Table 8. FAN in mash and backset: corn dry milling example[1].

	Usage level (L/1000 L)	*FAN (mg/L)*	*FAN contribution (mg/L)*
Backset	200	75	15*
Mash	~800	~88.4	70.7
		total	85.7

*quality questioned
[1]Ingledew, 1999

Table 9. FAN in backset and steepwater: corn wet milling example[1].

	Usage level (L/1000 L)	*FAN (mg/L)*	*FAN contribution (mg/L)*
Backset	436	75	32.7*
Light steepwater	98[2]	756	74
		total	107

*quality questioned
[1]Ingledew, 1999
[2]more is hard to add

NITROGEN SUPPLEMENTATION

The nitrogen content of a yeast cell varies between 3-9% (w/w) and one can thus make a rough calculation as to how much suitable nitrogen nutrient would be needed in a fermentation. If yeasts are ~6% N and we wish to grow 6 mg/mL of new yeast (~300 x 10^6 cells), then 0.36 mg/mL of usable N is needed. Thus if the source of N is urea, 770 mg/L is needed. If the source is NH_3, 440 mg/L is needed and if the source is diammonium phosphate (DAP), 1700 mg/L is needed. Tables 8 and 9 above illustrate the differences in useable FAN depending on the carbohydrate source and the use of backset and steepwater.

FAN AND YEAST GROWTH

Yeast growth increases almost linearly with FAN levels up to 100 mg/L. Very high amounts of FAN (up to 878 mg/L) have been shown to have no additional effect on yeast growth, however they did stimulate the fermentation rate.

$C_{3.72}\ H_{6.11}\ O_{195}\ N_{0.61}$ is the formula proposed for the major elements in yeast cell mass by Harrison (1967) and from this one can calculate the nitrogen content of a cell. This is of the order of 9% (w/w). The number is generally accurate for aerobically-grown yeast, but is an overestimation for yeast from anaerobic fermentations.

The level of protein inside a yeast is a function of the concentration of usable nitrogen (ammonium salts, urea, amino acids, di- or tripeptides and nitrogen derived from nucleic acid breakdown) (Figure 23).

SOURCES OF NITROGEN

Ammonium ion, supplied as ammonia or the sulfate or the phosphate salt, is a preferred nitrogen source for yeast cultivation in laboratory studies. When urea is utilized as the nitrogen source, biotin or other growth factors may be required. Urea, which is not a normal constituent of mashes, is easily broken down into two molecules of ammonium ion and one molecule of carbon dioxide. Many countries have now banned the use of urea as a yeast food ingredient for potable alcohol manufacturing because it leads to the production of small amounts of urethane (ethyl carbamate). This compound is a suspected carcinogen in foods (Ingledew *et al.*, 1987).

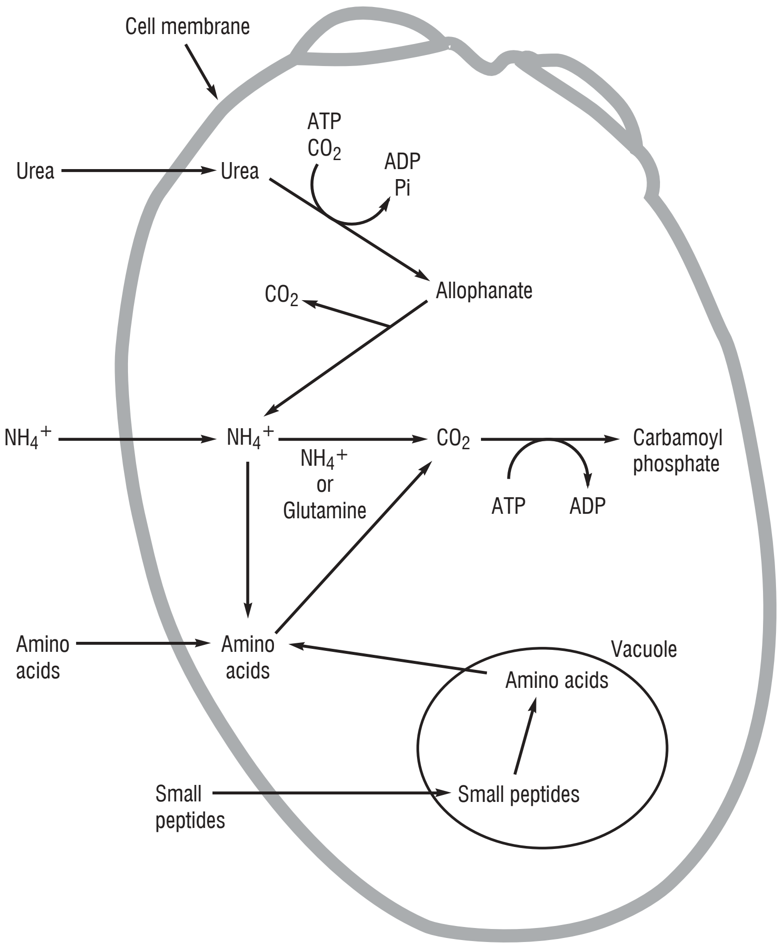

Cell membrane
Urea
Urea
ATP
CO2
ADP
Pi
Allophanate
CO2
NH4+
NH4+
CO2
Carbamoyl phosphate
NH4+ or Glutamine
ATP
ADP
Amino acids
Amino acids
Vacuole
Amino acids
Small peptides
Small peptides

Figure 23. Nitrogen uptake by the yeast cell (adapted from Slaughter, 2003).

For beverage alcohol the main source of nitrogen for yeast growth is amino acids formed from the proteolysis of grain proteins. The wort/mash contains 19 amino acids. The yeast takes up the amino acids in an orderly manner with different amino acids removed at various points in the fermentation cycle (Table 10). Proline, the most abundant amino acid in wort/mash, has scarcely been assimilated by the end of fermentation, whereas over 95% of the other amino acids have been used by the yeast.

There are a number of permeases for the uptake of wort amino acids, some specific for individual amino acids and one that is a general amino acid permease (GAP) with broad substrate specificity. The uptake and regulatory mechanisms are complex.

Although ammonia is a preferred nitrogen source for catabolic reactions, the cell will take up certain amino acids first for direct incorporation into proteins. The absorption of the amino acids by the yeast and their rate of assimilation will vary both with the strain of yeast and the amino acid composition of the wort/mash. Some amino acids such as glycine and lysine are readily taken up by the yeast. However, under nitrogen limiting conditions these two amino acids are inhibitory to growth and to fermentation. When usable nitrogen (such as other amino acids) is in excess, the glycine and lysine are not inhibitory. A balanced nitrogen source (i.e. a mixture of amino acids) is more efficient in providing nitrogen to the yeast for use in the various biochemical pathways than a single nitrogen source.

Table 10. Classes of wort amino acids in order of assimilation during fermentation[1].

Group A *Rapidly absorbed from start of fermentation*	*Group B* *Slowly absorbed from start of fermentation*	*Group C* *Slowly absorbed, late in fermentation*	*Group D* *Absorbed slowly over fermentation*
Aspartic acid/amine	Histidine	Alanine	Proline
Glutamic acid/amine	Isoleucine	'Ammonia'	
Lysine	Leucine	Phenylalanine	
Arginine	Methionine	Tryptophan	
Serine	Valine	Tyrosine	
Threonine		Glycine	

[1] Pierce, 1987.

NITROGEN DEFICIENCY

In regular gravity malt wort there is usually adequate amino nitrogen available for yeast growth but not when using adjuncts at high levels since many of these adjuncts are relatively deficient in amino nitrogen. When a starch slurry is used for fuel alcohol production, fermentation problems will result unless a nutritional supplement is added. The medium cannot support the growth required to ensure enough yeast biomass to catalyze the complete conversion of sugar to alcohol in a reasonable time frame. The addition of corn steep liquor, ammonia or other yeast foods is needed to ensure yeast growth. Molasses-based media can be deficient in 'yeast-usable' nitrogen. It can be supplemented with inorganic ammonium ion, urea or free amino acids. There may also be a deficiency in biotin, sulfur and phosphorus.

The addition of sugar syrups (compounds with no nutritive value other than the sugar content) dilute out the non-carbohydrate components while increasing the mash specific gravity. This can lead to a nitrogen deficiency that manifests itself in a sluggish or stuck fermentation (O'Connor-Cox and Ingledew, 1989).

Attention to the nutritional status of mash is important in order to prevent premature termination of fermentation. It is too late to provide nutrients or new yeast after fermentations have become stuck or sluggish.

YEAST FOODS

Yeast foods and fermentation supplements are designed to provide the lacking nutrients of fermentation media and have been successfully used for many years to enhance and ensure predictable fermentation times and product yields. Not only can they provide nitrogen, but depending on the formulation they can also alleviate vitamin and mineral deficiencies and provide other growth factors.

PROTEASE ADDITION

Yeasts used for ethanol production are not proteolytic and hence cannot break down proteins or utilize peptides much larger than tripeptides to obtain assimilable nitrogen for growth. Proteolysis, therefore, is intracellular unless the cells have lysed. Yeasts are only able to utilize low molecular weight nitrogenous materials such as inorganic ammonium ions, urea, amino acids and small peptides. When the yeast is starved for nitrogen, this will trigger catabolism of amino acids.

The basic amino acids are converted on entry into the cell to their N-free carbon skeletons. The deaminated carbon skeletons of hydrophobic amino acids are then excreted as α-keto acids and the corresponding fusel oils. Nitrogen starvation also leads to a small increase in protein degradation inside the cell.

Commercial proteolytic enzymes such as Allprotease™ can be used to create usable FAN from proteins and peptides in the mash. Figure 24 shows the benefit of protease addition. Where a plant is experiencing bacteriological problems, protease addition is recommended after yeast addition rather than before so that liberated amino acids are consumed by the yeasts rather than by the bacteria (Jones and Ingledew, 1994).

It is worth noting that surface culture enzymes such as Rhizozyme™ contain enough natural protease activity to make further additions unecessary.

Endogenous enzymes

In malt and grain distilleries, the mash, as distinct from the mash in a brewing operation, still has residual enzyme activity and a high proportion of the cereal starch is converted to fermentable sugars throughout the fermentation by continuing starch hydrolysis. In fuel ethanol fermentations, the addition of exogenous enzymes to break down starches performs the same function throughout the fermentation.

High gravity fermentation

High gravity fermentation employs worts/mashes at higher than normal concentration. High gravity fermentation is progressively being introduced around the world and yeast nutrition must be addressed when the change is made from regular gravity to high gravity. Sufficient oxygenation, higher nitrogen levels and higher yeast addition are the key success factors. Increased osmotic pressure and ethanol levels can affect the yeast and the stress tolerance of the yeast becomes a much more important factor. Stress tolerance during the fermentation of high gravity media has been shown to be very strain dependent. Although the issues of yeast nutrition in high gravity wort can be addressed by careful selection of strains and fermentation conditions, failure to address them can result in serious fermentation problems. Ingledew and coworkers have published extensively on the opportunities for very high gravity fermentation (VHG) and have extended the upper limits by successfully producing 23.8% (v/v) ethanol in a batch fermentation (Ingledew, 1999; Wang *et al.,* 1999a,b).

Stress factors and synergism

Stress factors such as ethanol, temperature, and acids can significantly impact the vitality and viability of the yeast. These factors can act synergistically and overpower the ability of the yeast to recover from stress. The list of stress factors is long and yeast has an amazing ability to recover from them – but there is a limit! It is important to remember that yeast is the heart of the fermentation process and if reasonable care and attention are given to ensure its health the payback will be huge.

‘Select and treat your yeast well and it will work well for you!’

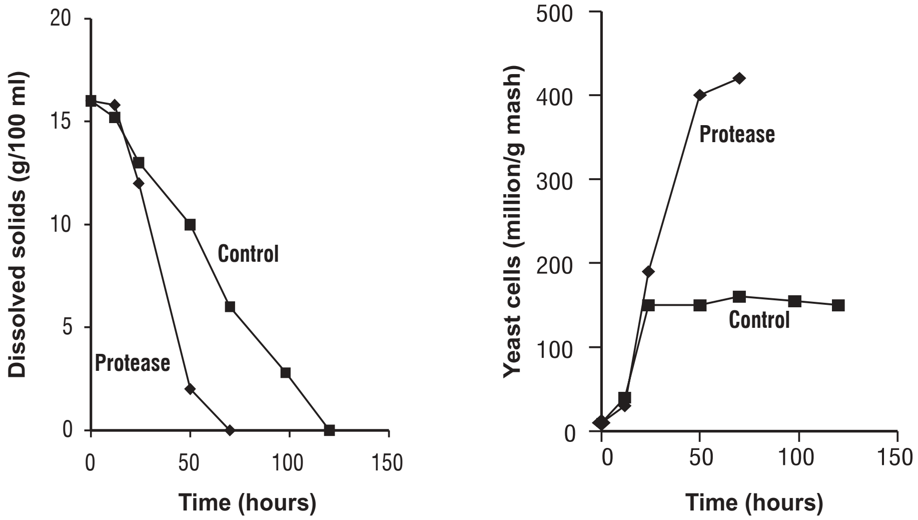


Figure 24. Benefits of protease addition to an alcohol fermentation (Thomas and Ingledew, 1990, by permission).

Acknowledgments

The author would like to acknowledge Dr. M. Ingledew and his coworkers (University of Saskatchewan) whose research work and previous publications are the foundation for this chapter, in particular their pioneering work in the fuel ethanol industry in the area of very high gravity fermentation and nitrogen requirements of yeasts. Acknowledgments are also due to Dr. G.G. Stewart (Heriot Watt University-International Centre for Brewing and Distilling.

References

American Society of Brewing Chemists. 1980. Report of the subcommittee on microbiology. J. Am. Soc. Brew. Chem. 38:109-110.

Austin, G.D., R.W.J. Watson, P.A. Nordstorm and T. D'amore. 1994. An online capacitance biomass monitor and its correlation with viable biomass. MBAA Technical Quarterly 31:85-89.

Barbour, E.A. and F.G. Priest. 1988. Some effects of *Lactobacillus* contamination in Scotch whisky fermentation. J. Instit. Brewing 94:89-92**.**

Campbell, I. 2003a. Wild yeasts in brewing and distilling. In: *Brewing Microbiology*, 3rd Edition. Kluwer Plenum, New York, pp. 247–266.

Campbell, I. 2003b. Yeast and Fermentation. In: *Whisky: Technology, Production and Marketing* (I. Russell, ed). Academic Press, London, pp. 117-154.

Dolan , T.C.S. 1976. Some aspects of the impact of brewing science on Scotch malt whisky production. J. Instit. Brewing 82:177-181.

Harrison, J.S. 1967. Aspects of commercial yeast production. Process Biochemistry. 2(3): 41-46.

Heggart, H.M., A. Margaritis, H. Pilkington, R.J. Stewart, T.M. Dowhanick and I. Russell. 1999. Factors affecting yeast viability and vitality. MBAA Technical Quarterly 36(4):383-406.

Heggart, H., A. Margaritis, R. Stewart, H. Pilkington, J. Sobczak and I. Russell. 2000. Measurement of yeast viability and vitality. MBAA Technical Quarterly 37(4):409-430.

Ingledew, W.M., L.A. Langille, G.S. Menegazzi and C.H. Mok. 1977. Spent brewers yeast – analysis, improvement and heat processing considerations. MBAA Technical Quarterly 14:231-237.

Ingledew, W.M., C.A. Magnus and J.R. Patterson. 1987. Yeast foods and ethyl carbamate formation in wine. American Journal of Enology and Viticulture. 38: 332-335.

Ingledew, W.M. 1999. Alcohol production by *Saccharomyces cerevisiae*: a yeast primer. In: *The Alcohol Textbook: A Reference for the Beverage, Fuel and Industrial Alcohol Industries* (K.A. Jacques T.P. Lyons and D.R. Kelsall**,** eds). Nottingham University Press, Nottingham, UK, pp. 49-87.

Jones, A.M. and W.M. Ingledew. 1994. Fuel alcohol production assessment of selected commercial proteases for very high gravity wheat mash fermentation. Enzyme Microb. Tech. 16:683-687.

Jones, R.P., N. Pamment and P.F. Greenfield. 1981. Alcohol fermentation by yeast-The effect of environmental and other variables. Process Biochem. 16(3):42-49.

Jones, R.P. and P.F. Greenfield. 1984. A review of yeast ionic nutrition - Part 1: Growth and fermentation requirements. Process Biochem. 19(2):48-60.

Maloney, D. 1998. Yeasts. In: *Kirk-Othmer Encyclopedia of Chemical Technology*, 4th Edition (J.I. Kroschwitz and M. Howe-Grant, eds). John Wiley & Sons, New York, pp. 761-788.

Neish, A.C. and A.C. Blackwood. 1951. Dissimilation of glucose by yeast at poised hydrogen ion concentrations. Canadian J. Tech. 29:123-129.

O'Connor-Cox, E.S.C. and W.M. Ingledew. 1989. Wort nitrogenous sources – Their use by brewing yeasts: a review. J. Amer. Soc. Brewing Chem. 47:102-108.

Patel, G.B. and W.M. Ingledew. 1973. Internal carbohydrates of *Saccharomyces carlsbergensis* during commercial lager brewing. J. Inst. Brewing 79:392-396.

Peppler, H.J. 1970. Food yeasts. In: *The Yeasts*, Vol 3, Yeast Technology. Academic Press, New York, pp. 421-461.

Pierce, J.S. 1987. The role of nitrogen in brewing. J. Instit. Brewing 93:378-381.

Reed, G. and T.W. Nagodawithana. 1991. In: *Yeast Technology*, 2nd Edition. AVI. Van Nostrand Reinhold, New York.

Slaughter, J.C. 2003. The biochemistry and physiology of yeast growth. In: *Brewing Microbiology, 3rd edition*. Kluwer Plenum, New York. pp. 19-62.

Smart, K.A., K.M. Chambers, I. Lambert and C. Jenkins.1999. Use of methylene violet staining procedures to determine yeast viability and vitality. J. Am. Soc. Brew. Chem. 57(1):18-23.

Smart, K. 2000. The death of the yeast cell. In: *Brewing Yeast Fermentation Performance*. Blackwell Science, United Kingdom, pp. 105-113.

Thomas, K.C. and W.M. Ingledew. 1990. Fuel alcohol production: effects of free amino nitrogen on fermentation of very high gravity wheat mashes. Appl. Environ. Microb. 56:2046-2050.

Walker, G. 1999. *Yeast Physiology and Biotechnology*. John Wiley and Sons Ltd. United Kingdom.

Wang, S., W.M. Ingledew, K.C. Thomas, K. Sosulski and F.W. Sosulski. 1999a. Optimization of fermentation temperature and mash specific gravity for fuel alcohol production. Cereal Chem. 76:82-86.

Wang, S., K.C. Thomas, K. Sosulski, W.M. Ingledew and F.W. Sosulski. 1999b.Grain pearling and the very high gravity (VHG) fermentation technologies for fuel alcohol production from rye and triticale. Process Biochemistry. 34(5): 421-428.

Recommended reference books

The following books were invaluable in the preparation of this chapter and are highly recommended to anyone wishing to gain more in depth knowledge on yeast.

An Introduction to Brewing Science and Technology, Series III, Brewers Yeast.1998. G.G. Stewart and I. Russell. Published by the Institute and Guild of Brewing. Available from www.igb.org.uk.

Biotechnology of Malting and Brewing. 1991. J.S. Hough, Cambridge University Press.

Brewing Microbiology. 2002. F.G. Priest and I. Campbell. (eds). Kluwer Academic.

Beers and Coolers. 1994. Manfred Moll. Springer-Verlag, New York.

Brewing Yeast and Fermentation. 2001. C. Boulton and D. Quain. Blackwell Science.

Brewing Yeast Fermentation Performance. 2000. 1st Edition. K. Smart (ed). Blackwell Science.

Brewing Yeast Fermentation Performance. 2003. 2nd Edition. K. Smart (ed). Blackwell Science.

Handbook of Brewing. 1994. W. A. Hardwick. Marcel Dekker.

Standards of Brewing. 2002. C.W. Bamforth. Brewers Publications.

Technology - Brewing and Malting, 2nd Edition. 1999. W. Kunze. Verlag der VLB Berlin. Available from the VLB at www.vlb-berlin.org/english/books/kunze/.

The Alcohol Textbook: A Reference for the Beverage, Fuel and Industrial Alcohol Industries. 1999. K.A. Jacques, T.P. Lyons and D.R. Kelsall (eds). Nottingham University Press, Nottingham, UK.

The Practical Brewer [MBAA], 3rd Edition. 1999. Available from the Master Brewers Association of the Americas at www.mbaa.com.

Whisky: Technology, Production and Marketing. 2003. I. Russell (ed). Academic Press, New York.

Yeast Physiology and Biotechnology. 1998. G. Walker. John Wiley and Sons Ltd., New York.

Chapter 10

Practical management of yeast: conversion of sugars to ethanol

DAVE R. KELSALL AND T. PEARSE LYONS

Alltech Inc., Nicholasville, Kentucky, USA

Introduction

Fermentation is the critical step in a distillery. It is here that yeast converts sugar to ethanol; and it is here that contaminating microorganisms find an opportunity to divert the process to lactic acid, glycerol, acetic acid, etc. The fermentation step (and to a much lesser degree the cooking process) is also where yeast must be 'coached' to produce maximum levels of ethanol, sometimes as high as 23%. Fermentation will also have a direct bearing on downstream processing. If sugar levels remaining in beer are too high, evaporative capacity may be impaired or syrup contents increased. Both can lead to distillers grains being more difficult to dry and altered in color as Maillard reactions occur between proteins and sugars. Truly the fermentation step is the heart of the distillery.

When reflecting on the overall flow diagram of the distillery (Figure 1) the central role of yeast is obvious. The process of converting sugars to ethanol takes between 10 and 60 hrs during which heat is generated. The various factors affecting the efficiency of that process need to be considered. In the chapters by Russell and Ingledew, the biochemistry of ethanol production by yeast is covered in detail. In this chapter we will discuss the factors involved in taking liquefied mash from cooking (1-2% glucose) to ensure that pre-formed sugar and other bound sugars (starch and other polysaccharides) are released in a timely fashion for maximum conversion to ethanol by yeast.

Choosing a yeast strain

One of the most important decisions a distiller must make is the selection of yeast strain. The best characterized yeast is *Saccharomyces cerevisiae*, but within this species there are thousands of unique strains. Which one to use? Several decisions must be made (Table 1). The first decision is whether or not to grow the strain in-house. In fact, yeast strain maintenance and growth is a job best left to the experts. For the very small cost per gallon it represents (less than 0.5 cents), there is just too much at stake.

Table 1. Choices among yeast strains.

- Grow in-house?
- Active dried or pressed?
- High alcohol tolerance needed? Thermosacc™
- Lower alcohol in fermentor: Superstart™

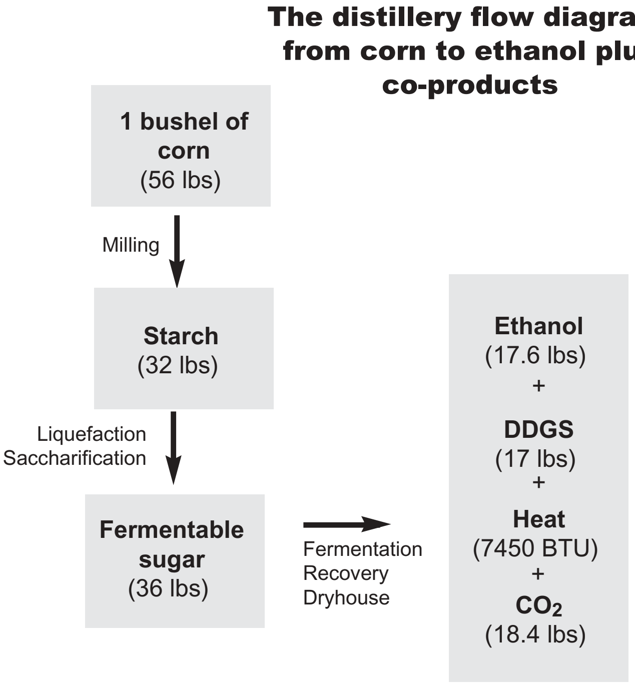

The distillery flow diagram: from corn to ethanol plus co-products
1 bushel of corn (56 lbs)
Milling
Starch (32 lbs)
Liquefaction
Saccharification
Fermentable sugar (36 lbs)
Fermentation
Recovery
Dryhouse
Ethanol (17.6 lbs)
+
DDGS (17 lbs)
+
Heat (7450 BTU)
+
CO_2 (18.4 lbs)

Figure 1. Distillery product flow.

ACTIVE DRY OR PRESSED YEAST?

The choice between active dried (95% solids) or pressed yeast (35% solids) very often comes down to two points: adequate cold storage (pressed yeast must be stored at 5°C and has a shelf life of 1-2 months) and whether the yeast must 'start immediately'. Yeast requires time to adapt to any new environment and activate its metabolism, but first must be rehydrated. This is a period of zero growth but intense biochemical activity. If we are conditioning the yeast, the advantage of a pressed yeast is that the lag phase is minimized because the yeast is already hydrated. Cell numbers are typically 7-10 billion cells/g. Active or instant dry yeast (ADY) are vacuum packed, often under nitrogen, and are easy to store with minimal losses in activity (5% over 6 months at 5°C). ADY requires wetting, and the lag phase tends to be 1-2 hrs. Cell numbers 15-20 billion cells/g.

IS HIGH TEMPERATURE AND/OR HIGH ALCOHOL TOLERANCE NEEDED?

Yeast is sensitive to temperature, high or low, and responds by activating stress mechanisms including elevation of intracellular trehalose. Additionally, certain proteins, called heat shock proteins, are synthesized. Some strains are better able to withstand temperature stress than others. Such yeast are also usually able to tolerate higher alcohol levels. In the 1980s it was customary to run fermenters at 8-10% ethanol but now 16-17% is the norm. One yeast, Thermosacc™ , is a good example of a stress resistant strain. Smaller in size (5 µm instead of 8-10 µm, Figure 2), it has a temperature tolerance of >40°C and an ethanol tolerance in excess of 20%. An unusual feature is its ability to tolerate high acetic and lactic acid levels. This may explain observations of 'cleaner' fermentations as infecting microorganisms such as *Lactobacillus* have less impact. In beverage alcohol production, little, if any, change in congeners has been reported following its use.

Controlling yeast stress factors that affect alcohol yield

TEMPERATURE: THE FIRST STRESS FACTOR

Inability to precisely control the temperature of

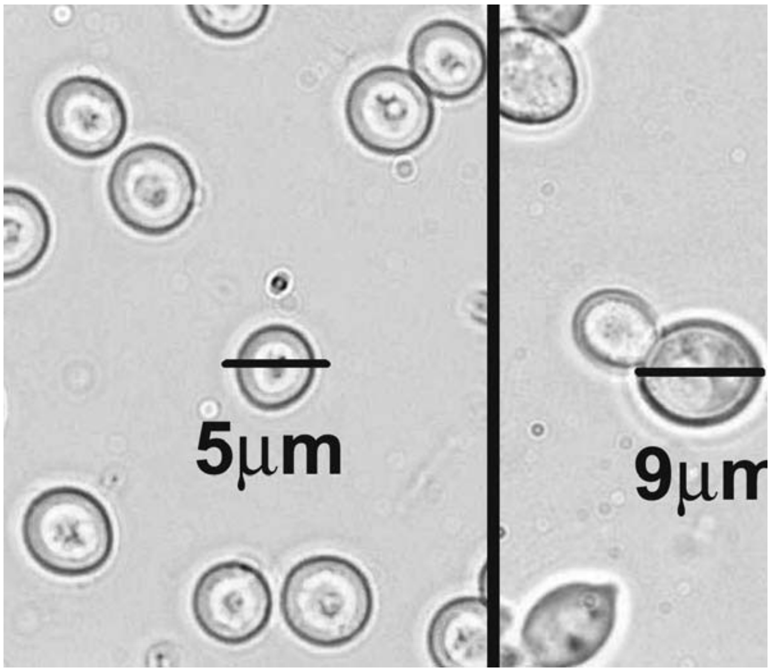


Figure 2. Thermosacc™ *vs* regular yeast.

fermentation is possibly the biggest and most commonly encountered factor or problem affecting alcohol yield. Almost all distilleries suffer from underestimation of the amount of energy released during fermentation. Consider the general conversion of glucose to alcohol as illustrated in Figure 1. Generation of most of the energy of fermentation of ethanol takes place between hours 10 and 30 of fermentation. In other words, the fermentor cooling system should be designed to cool 44,000 BTU per 100 lbs of ethanol produced over a 20 hr period. Often this amount of cooling is not designed into the system and the fermentors become overheated. Engineers did not anticipate the enormous strides that would be made in yeast fermentation (higher alcohol tolerance, faster conversion) and designed for shorter residence times and less cooling capacity.

The theoretical temperature rise for a 25 gallon beer (25 gallons of water per bushel of grain) is 22ºC per bushel (Figure 3). The optimum temperature of fermentation for yeast *(Saccharomyces cerevisiae)* is 35°C and the optimum temperature of reproduction for the yeast is 28°C (yeast capable of reproducing at 35°C is now available). It can also be seen (in Figure 3) that without adequate cooling, a fermentor set at 35ºC would inevitably increase in temperature. Every degree in temperature above 35°C will have a tendency to depress fermentation, as the yeast cannot handle these higher temperatures. Furthermore, the increase in temperature favors the growth of lactobacilli,

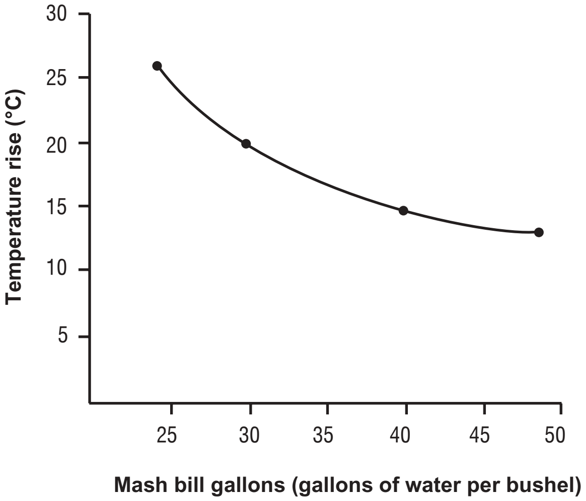


Figure 3. Theoretical temperature increase during fermentation.

the microorganism that competes with yeast for glucose. Heat also increases the impact of other stress factors such as mycotoxins and salt levels.

Combining enzyme activities to control sugar delivery to yeast

How can we best control fermentation temperature? The easiest way to reduce fermentor temperature would be to reduce the sugar level going in and therefore reduce yeast growth; however, less alcohol would be produced. Since the objective is more, not less alcohol, this is not an option. However, sugar level, or more importantly sugar type, is important.

Yeast must be maintained in a growth (budding) phase, since their ability to produce alcohol is more than 30 times greater during growth than in non-growth mode. Yeast however can respond to high glucose levels in two ways. Cell growth is either inhibited by high glucose levels or yeast may grow rapidly and then stop. In either case there is an over-dosing effect on fermentation efficiency and possibly a spike in temperature. Glucose must be 'spoon-fed' to the yeast; and for this reason it is recommended that glucoamylases be used both sparingly and in conjunction with an enzyme produced in surface culture called Rhizozyme™. The combination provides ideally balanced sugar delivery to maintain good yeast growth and maximum alcohol productivity while avoiding high temperature peaks. Glucoamylase (Allcoholase II L400) provides glucose at the start of fermentation while the Rhizozyme™, with pH and temperature optimums more closely aligned to fermentation conditions, continues to work during fermentation. The net result is a steady, slow release of glucose and a steady formation of alcohol by yeast.

Rhizozyme™ also affects ethanol yield as it is capable of hydrolyzing starch that is unconverted in the cook process and also converts some of the cellulose in the corn to glucose.

The key is to consider cooking and fermentation together. Cooking must be designed to release as much starch, both bound and unbound, as possible without overloading the yeast with glucose. It must also be recognized that corn has, in addition to starch, other potentially useful carbohydrates including cellulose and hemicellulose. Conventional glucoamylases do not degrade either, whereas glucoamylase produced by surface culture fermentation (Koji) also contains a range of carbohydrase enzymes as 'side activities' that degrade some of the cellulose thereby releasing more sugar, which boosts yield (Table 2, Figure 4).

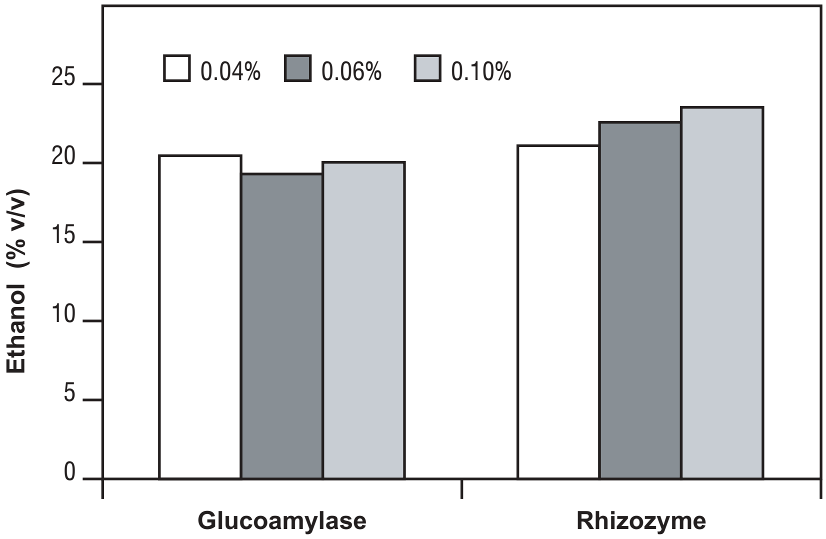


Figure 4. Comparison of alcohol yields from high solids corn mash with traditional and surface culture glucoamylases.

Table 2. Comparison of surface culture and conventional glucoamylase.

Production	*Surface culture (Koji) fermentation*	*Conventional deep tank liquid*
Activities		
Glucoamylase	40	400
Cellulase	400	—
Hemicellulase	350	—
α-amylase	5000	—
Phytase	300	
pH profile	Broad	Narrow
Temperature optimum, °C	30-40	0-60
Activity on gelatinized starch	Rapid	Slow
Activity on raw starch	Reasonable	Slow
Yeast stimulation	Significant	Poor

Modeling provides a prediction of the increase in yield to be expected. Predicted yields of 3.1 gallons per bushel, 17-18% higher than the standard 2.65, have been calculated based on increased sugar release. This is 18 gallons (>65 liters) more per tonne of grain. The recommendation is to use a 50/50 blend of Rhizozyme™ Koji and conventional glucoamylase to maximize yield. Where distillers can reliably measure yield, the results in terms of costs per gallon reveal optimum returns.

Equipment for heat control

Despite the sequential feeding of glucose, heat is generated during fermentation and must be removed. Four types of heat exchangers are typically used; and these are illustrated later in this chapter when fermentation design is discussed.

a) *Internal cooling coils*. These are the least desirable option because they are practically impossible to clean and sterilize.

b) *Internal cooling panels*. Like cooling coils, panels are suspended in the fermentation vessel. Also like cooling coils, cooling is adequate but sterilization may be difficult.

c) *External cooling jacket*. Cooling jackets, often dimpled for added contact with the fermentation, can cover either part of the vessel or the entire vessel. Recirculating coolant through the jacket allows fermentation temperatures to be monitored. If jackets are spaced out over the fermentor sides, then cooling can commence as the fermentor is filled.

d) *Heat exchanger with recirculation*. Contents of the fermentor are pumped through a heat exchanger (typically spiral or plate-and-frame) and returned to the fermentor. This has the added advantage of acting as a means of keeping the fermentor agitated; and nutrients (sterol donors, peptides, oxygen, etc.) can be introduced at critical times. It is suggested that optimum control of temperature can be obtained by placing the heat exchangers in such a position that recirculation can start at the beginning of the fill when the yeast starts producing heat.

Of the four types, the cooling jacket is best for minimizing infection, but the heat exchanger is the most effective at heat control. As the distiller moves towards very high levels of ethanol (over 20% by volume), heat recovery and maintenance of low temperatures will become even more important as temperature rises of 6-8°C will become the norm. A word of caution: some distilleries, particularly in continuous operations, share external heat exchangers between fermentors in order to economize. Based on the experience of many existing distilleries using this sharing concept, infection can easily be transferred among fermentors as well as having inadequate cooling.

INFECTION: THE SECOND STRESS FACTOR

The various types of infection are described in detail elsewhere in this book, however several points are relevant in this context. Lactobacilli consume glucose to produce lactic acid and acetic acid, which is the second major factor affecting the yield of alcohol in fermentation, which in turn has a major impact on distillery economics (Table 3). Yeast do produce some organic acids during fermentation, but concentrations are relatively low compared to those produced by lactobacilli and other contaminating bacteria. As a general rule of

Table 3. Relationship between bacterial numbers and ethanol production losses.

Viable bacteria in mash at set (CFU/ml)	*Ethanol lost[1] (% v/v)*	*Ethanol lost[2] (gallons)*	*Financial loss[3] (in US dollars)*
105	0.1 - 0.2	80,000	112,000
106	0.2 - 0.4	160,000	224,000
107	0.6 - 1.0	400,000	560,000
108	0.9 - 1.2	480,000	672,000
109	1 - 1.5	600,000	840,000

[1] Depending on the strain of Lactobacillus.
[2] Maximum loss calculated based on a 40,000,000 gallon/yr.
[3] Calculated based on ethanol price of $1.40/gal.

thumb, if the titratable acidity of an uninoculated mash is X, then the titratable acidity of the fermented beer is usually about 1.5X. This is a general rule and seems to work quite well in practice, although it is not based on known scientific principles. The 1.5X represents the titratable acidity of the finished beer when there is no significant contamination from lactobacilli. When lactobacilli are active, the generation of lactic and acetic acids substantially increases the titratable acidity and often the high acid content will cause the yeast fermentation to stop or dramatically slow down. Very early into an infection the titratable acidity reaches 2X.

Practical means of controlling infection

The most fundamental way to control infection is to control the fermentation environment (Table 4). Fermentors must be cleaned on a regular basis; and designs must avoid sharing of heat exchangers and dead legs in piping. Fermentors should be designed to facilitate complete emptying. Scaling will occur regardless of the fermentor design, and should be removed using either a scale inhibitor (such as Scale-Ban™) or a de-scaling chemical (an acid wash). Fermentations must be fast; and yeast should be added either in a form that will quickly rehydrate or preferrably one that has been ‘started’ in a conditioning tank. Saccharification tanks can be a source of infection as lactobacillus strains can adapt and grow at the temperatures used in this process. The goal is to make conditions as suitable as possible for yeast growth (without jeopardizing alcohol yield) and as unsuitable as possible for infectious microorganisms. By restricting glucose release in fermentation by using Rhizozyme™ and lower levels of glucose-releasing glucoamylase (typically 50-70%), the yeast uses the sugar as it becomes available and competitively excludes lactic and acetic acid-producing bacteria by reducing available sugar. Equally, when yeast growth rate begins to decline (typically at 20-24 hrs) sterol donors and oxygen, which at that stage have become limiting, will revive activity by ensuring that fatty acids are available for yeast reproduction.

Table 4. Practical means of controlling infection.

Avoid deadlegs in lines
Avoid sharing fermentor heat exchangers
Proper cleaning programs
  Periodic use of de-scaling chemicals
Control fermentation temperatures
  Avoid the saccharification step in cooking
Maximize yeast growth (budding)
  Use yeast yeast conditioning
  Use sterol donors and peptides as yeast foods in yeast conditioning
Antimicrobials
  Allpen™ (against Gram-(+) organisms)
  Lactoside™ (against Gram-(+) and Gram-(-) organisms)

Regardless of precautions, a distillery is not a sterile environment and opportunities for growth of both Gram (+) and Gram (-) bacteria exist. Because their growth rate is so much faster than yeast, strict controls to prevent infection must be in place. As early as 1954 penicillin was used in ethanol fermentation; and owing to its quick inactivation caused no problems with regulatory agencies. Other antibiotics such as virginiamycin are effective antimicrobials, but some are now known to suppress yeast fermentation and in addition can leave residues (2.6%) in spent grains. With the ban in Europe against use of virginiamycin in products destined for animal feeds, caution is urged. Furthermore, resistance to both penicillin and virginiamycin is increasingly common.

One of the strategies to avoid this risk is to take advantage of the synergy that occurs when antimicrobial substances are combined, which lowers the amounts used. Lactoside™, a combination of antimicrobials without virginiamycin, has proven very effective for this reason. A routine maintenance dose of 1-2 ppm is practical as a precautionary measure. When there is infection the dosage is increased to 2-5 ppm. There is no evidence of carryover of these antibiotics into spent grains and solubles.

ALCOHOL LEVELS: A THIRD STRESS FACTOR

High alcohol beers have a tendency to stop fermenting. Understanding how alcohol levels act to stress yeast requires a working knowledge of yeast metabolism and growth. It has been shown that when yeast are in the reproductive phase they produce alcohol over 30 times faster than when not reproducing. Figure 5 shows typical yeast growth patterns or phases during fermentation. The first phase is the so-called lag phase, during which the yeast adapt to the fermentor environment. During this period there is little or no yeast growth and consequently little or no alcohol production. This period can last from 4 to 12 hrs. Ways of managing and reducing the length of the lag phase are shown in Figure 6 in reference to yeast conditioning.

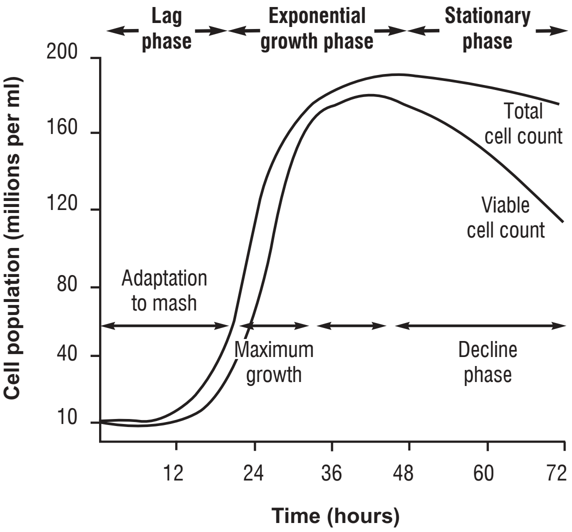


Figure 5. Typical yeast growth curve in distillery mash.

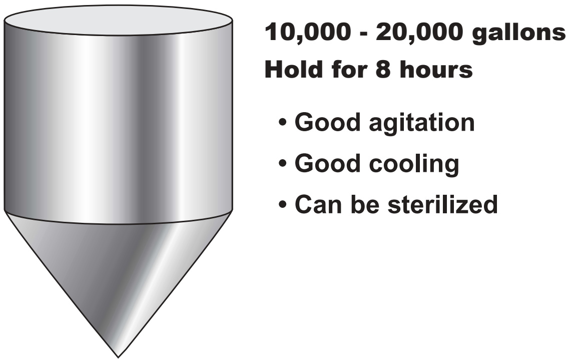


Figure 6. Yeast conditioning tanks: A key factor in maximizing yeast cell numbers and improving alcohol levels.

The second phase is the exponential growth phase. This is the most important phase where nearly all of the alcohol is produced. There is a limited time in which the yeast stay in this phase; and this is the factor limiting the quantity of alcohol produced during a given fermentation. The length of time during which the yeast can remain reproducing depends on the nutrition available in the fermentor.

The three major factors that significantly affect alcohol yield are therefore temperature, acidity of the mash and the alcohol level produced during fermentation. Yeast is tremendously resilient and usually produces efficiently despite all the negative conditions imposed. Yeast fermentation will yield well if the temperature is a few degrees high or if the acidity is a little high indicating a mild infection. Beers containing up to 20% alcohol v/v can be produced without slowing fermentation. However, when changes in two or more of these factors happen simultaneously, serious losses in yield usually occur. For example, in a mash at 37°C contaminated with lactobacilli at 10 million CFU/ml, it is likely that fermentation would stop at 8% alcohol, a 33% loss in yield.

MAXIMIZE YEAST PERFORMANCE BY CONDITIONING

Yeast is the powerhouse of any distillery; and without healthy yeast, alcohol percentages in the fermentor and alcohol yield will drop. Conditioning tanks are a way to ensure good cell numbers in the fermentor (Table 5). At the start of batch fermentation there should be a minimum of 50 million cells/ml, which should increase to 150-200 million cells/ml at the height of fermentation and drop to 100 million cells/ ml at the end. Continuous fermentors should maintain cell numbers of 150 million from a high of 250-300 million cells/ml. Yeast viability as measured by methylene blue stain should be 98-99% in the conditioning tank and 90% at the start of fermentation. There will be a drop to around 50% viability at the end of fermentation. In a cascade continuous fermentation system, viability was seen to drop to 30% in the last fermentor.

Table 5. Keys to a good yeast conditioning tank (pre-fermentor).

- Keep Brix to 10-20°, preferably ~14°Brix
- Provide good agitation
- Use peptide based yeast food
- Achieve minimum of 200 ppm FAN (free amino nitrogen)
- Add Rhizozyme™ to 'spoon-feed' yeast glucose (0.05% of mash)
- Hold for 4-8 hrs with agitation
- Achieve 300-500 million cells/ml before transfer to main fermentor
- Pump to fermentor
- Clean and sterilize
- Repeat

A typical charge for a continuous yeast conditioning tank is shown in Table 6. The mash was sterilized and cooled to 90°F. Yeast was added with good agitation along with yeast food and saccharifying enzyme (Rhizozyme™). The mash was held for 8 hrs at which stage a cell count in excess of 500 million was achieved. The mash was transferred to the fermentor and the tank cleaned and sterilized. In this way distillers ensure good yeast growth with minimum 'lag phase' periods.

Table 6. Typical charge for yeast conditioning tank for a 500,000 gallon fermentor.

Prefermentor size	20,000 gallons
Mash	Direct from jet cooker: dilute to 14°Brix
Active yeast	200 lb Thermosacc™
Peptide yeast food	20 lbs AYF
Rhizozyme™	1 lb
Yeast cell numbers expected	400-500 million
Time	8 hrs

MYCOTOXINS: A STRESS FACTOR

In 1985 the Food and Agricultural Organization (FAO) of the United Nations estimated that at least 25% of the world's grain supply is contaminated with mycotoxins. The type and degree of contamination depend on many factors including conditions during growth and harvest and geographic area. Toxins such as zearalenone and vomitoxin (deoxynivalenol) produced by growth of Fusarium molds in stored grain tend to be the mycotoxins of concern in the more temperate climates of North America and Europe while aflatoxin, produced by various species of Aspergillus, predominates in tropical climates and can be a serious factor in parts of the US. However a wide range of toxins and mixtures of toxins are usually present and all are of concern in the animal feed and human food chain. To a distiller, the main concern with mycotoxins is whether they pass through into the DDGS. The FDA have set definitive limits on aflatoxin levels for interstate grain shipments; and the increasing knowledge of the variety of mycotoxins and extent of contamination possible have heightened concern in all of the animal feed industry.

Of more recent concern to the distiller has been the finding that mycotoxins can also affect yeast growth (Table 7). Perhaps presence of these toxins may provide at least part of the reason behind unexplained unfinished fermentations. Since not all mycotoxins are destroyed by heating, it is unlikely that the cooking step would remove or inactivate them. In animals many of the mycotoxins damage protein metabolism causing reduced growth and increased disease susceptibility. Research into ways of nutritionally compensating for mycotoxin presence in animals feeds led to discovery that yeast cell wall glucomannans have characteristics that make them very specific adsorbents for certain mycotoxins. For example, about 80% of the zearalenone in solution can be bound and inactivated by adding yeast glucomannans. These products are also beginning to be used successfully in the fermentation industry.

Table 7. Effects of mycotoxins on yeast growth.

Mycotoxin	*Level required to inhibit yeast growth (ppm)*
Zearalenone	50
Vomitoxin	100
Fumonisin	10

PHYTIC ACID: AN ANTINUTRITIONAL FACTOR

Phytic acid, the form in which 60-80% of the phosphorus is stored in cereal grains, is a stress factor for yeast that may be converted into a nutritional benefit. The phytic acid molecule has a high phosphorus content (28.2%) and large chelating potential. In addition to holding phosphorus in tightly bound form, phytic acid can form a wide variety of insoluble salts with di- and trivalent cations including calcium, zinc, copper, cobalt, manganese, iron and magnesium. This binding potentially renders these minerals unavailable in biological systems; and makes phytin content a familiar 'antinutritional factor' when cereal grains are fed to livestock. Animal nutritionists either compensate for the presence of phytin with extra-nutritional levels of zinc, calcium and phosphorus or add microbial phytases to diets for pigs and poultry.

Phytin may also have an antinutritional effect on yeast. Calcium ion concentration affects enzyme activity; and minerals such as zinc are needed for yeast growth. Phytic acid is also known to inhibit proteolytic enzymes; and phytate-protein or phytate-mineral-protein complexes may reduce the breakdown of protein to amino acids required by yeasts for growth. Starch is also known to be complexed by phytate. Finally, inositol, a limiting nutrient for yeast growth, forms a part of the phytin molecule. The addition of a phytase enzyme (often present naturally in Koji surface culture enzyme sources) can overcome this problem.

SUMMARY: STRESS FACTORS AFFECTING YEAST ACTIVITY

As we 'coach' yeast to produce very high levels of ethanol, it becomes of paramount importance that we both understand the stress factors involved and design equipment to allow us to control them (Figure 7). Critical stress factors such as temperature, alcohol levels and infection, while understood individually, combine to produce unique conditions in every fermentation. Such conditions will vary with feedstocks used, but also with changes in the composition of individual feedstocks due to seasonal or climatic variables. An understanding

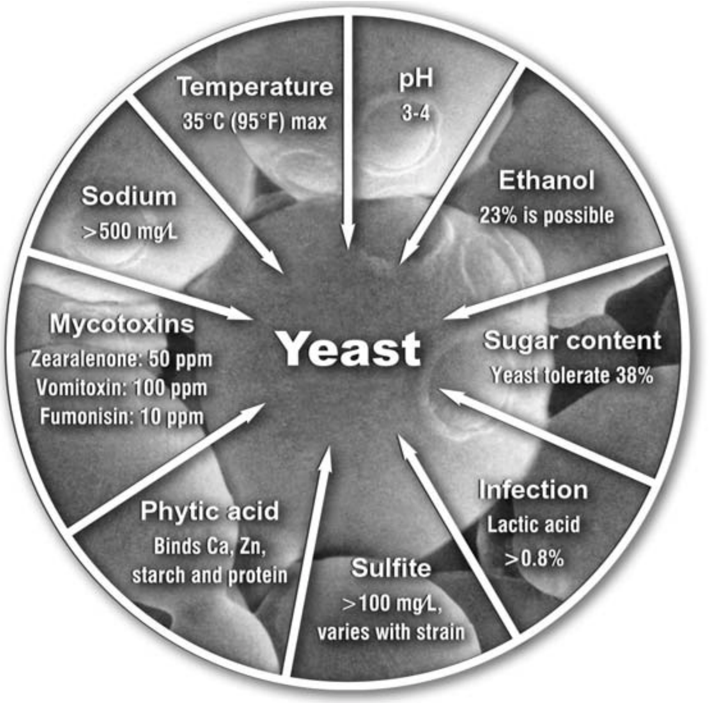

Temperature
35°C (95°F) max
pH
3-4
Ethanol
23% is possible
Sugar content
Yeast tolerate 38%
Infection
Lactic acid
>0.8%
Sulfite
>100 mg/L,
varies with strain
Phytic acid
Binds Ca, Zn,
starch and protein
Mycotoxins
Zearalenone: 50 ppm
Vomitoxin: 100 ppm
Fumonisin: 10 ppm
Sodium
>500 mg/L
Yeast

Figure 7. Stress factors affecting yeast.

of other feedstock-related stress factors such as mycotoxins or phytin content will also become more important as we move toward higher ethanol production.

Fermentation systems used in distilleries

Before considering the specific management tools available to the distiller, it is necessary to consider the differences among the fermentation systems in use. Over the last 30 years the brewing and distilling industries have developed new fermenting systems; and the distilling industry has largely converted to rapid batch fermentation with cylindro-conical or sloping bottom fermentors, or to cascade continuous fermentation using cylindro-conical fermentors.

BATCH FERMENTATION

A typical cylindro-conical fermentor with skirt support is illustrated in Figure 8. These fermentors are usually designed to ferment from 30,000 gallons to 750,000 gallons. Fermentors need to be fabricated on site if the diameter and length exceed a size that can be safely transported by road.

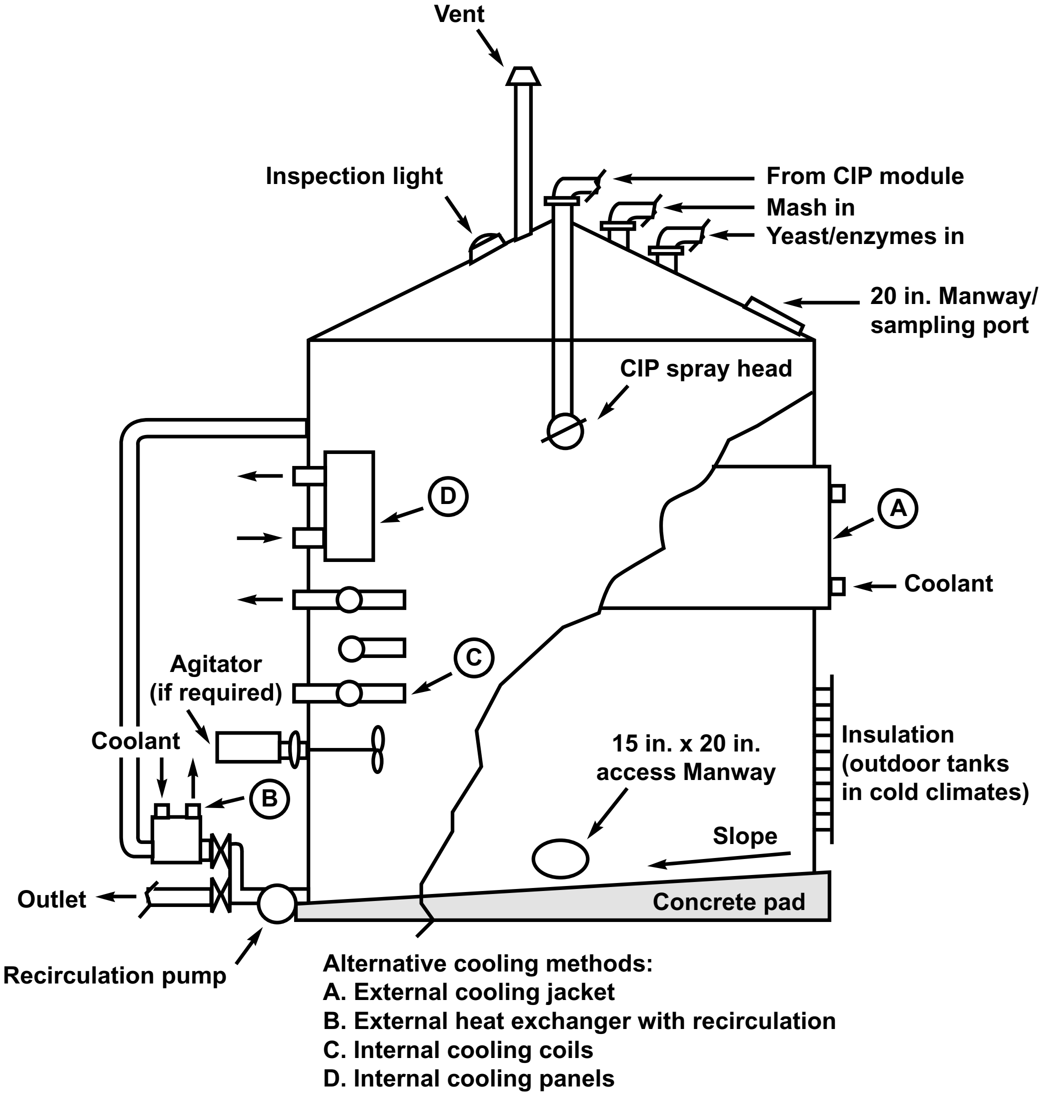

Vent
Inspection light
From CIP module
Mash in
Yeast/enzymes in
20 in. Manway/ sampling port
CIP spray head
D
A
Coolant
C
Agitator (if required)
Coolant
B
15 in. x 20 in. access Manway
Insulation (outdoor tanks in cold climates)
Slope
Outlet
Concrete pad
Recirculation pump
Alternative cooling methods:
A. External cooling jacket
B. External heat exchanger with recirculation
C. Internal cooling coils
D. Internal cooling panels

Figure 8. Alcohol fermentor with sloping bottom (Alltech/Bishopric, 1981).

Different cooling systems are available. Many distilleries use chilled water to cool the fermentors. From a microbiological standpoint the most desirable cooling system by far uses external cooling jackets. The least desirable option employs internal cooling coils because they are practically impossible to clean and sterilize from the top-mounted CIP (clean-in-place) spray nozzles. The external recirculation heat exchanger is frequently used in distilleries and is sometimes shared with other fermentors. This is a poor option as there are times when more than one fermentor needs to be cooled. The main disadvantage of this system when shared among fermentors is bacterial cross-contamination. With such a system, the microbiological condition of all fermentors will be determined by the fermentor with the most contamination. Even when these recirculating heat exchangers are not shared, they are difficult to clean. Throughput of at least 7 ft^3/sec must be obtained to ensure turbulent flow through the tubes.

The agitator is required particularly at the start and at the end of fermentation. Frequently the agitators are badly designed and provide poor mixing. A folding action is required to ensure proper mixing of the mash solids and to ensure an even temperature throughout the fermentor.

Carbon dioxide (CO_2) is removed through the vent. Fermentors must be fitted with pressure relief valves and vacuum breakers to avoid serious accidents. The CO_2 is frequently collected and sold. In any case, CO_2 should be scrubbed to remove alcohol, which is returned to the beer well. The CO_2 header manifold can be a source of contamination as infected mash from one fermentor can migrate in the CO_2 into another non-contaminated fermentor. The CO_2 manifold should be designed to be cleanable.

The lag phase is a great opportunity for lactobacilli to become established. Bacteria under optimum growth conditions can reproduce every 20-30 minutes; however yeast, which are much larger microorganisms, can only reproduce every 3 hrs. A single bacterium reproducing every 20 minutes would produce a population of 256 in 3 hrs. Some yeast strains have lag phases as long as 12 hrs, during which time the lactobacilli can become heavily established. Therefore, it is very important to choose a yeast strain with a very short lag phase and to use a conditioning tank so that the main fermentation starts immediately after the yeast is added.

There is a choice of materials to use in the manufacture of fermentors. Stainless steel is generally the best choice as it is easier to clean and sterilize. It also lasts much longer than mild steel or lined vessels; and when considered over the expected lifetime of a fermentor it is by far the most economical option. If chloride levels are high, then special stainless steels should be considered to avoid stress corrosion.

It is important that the slope of the bottom be sufficient for the mash to run out when discharging. This type of fermentor is much better suited for use with clear or semi-clear mashes than with whole cereal mashes.

Fermentor cleaning is accomplished with clean-in-place (CIP) equipment. CIP sprayheads are high-pressure devices (100-120 psi), which usually have automated cleaning cycles. A typical cleaning cycle would be (minimum):

Pre-rinse with water	10 minutes
Detergent circulation	20 minutes
Post-rinse with water	10 minutes
Sterilization	10 minutes

The detergent used would be based on caustic soda, normally with added wetting agent, antifoam and de-scaling agent. The caustic strength should normally be in the 3-5% range. Ideally, the detergent should be hot (80-90°C). The detergent and rinse waters should be continually drawn off from the fermentor to prevent accumulation of liquid during the cycle. The detergent and post-rinse should at least be recirculated and continually made up to strength. Chlorine dioxide and iodophors make ideal sterilizing agents, but many distillers still use steam to sterilize the fermentors. This is time-consuming and is probably not as effective as chemical sterilization.

It is important to control the build up of beerstone (calcium magnesium phosphate and calcium oxalate). Bacteria can penetrate beerstone, which is insoluble in straight alkaline solutions. Beerstone thus protects the bacteria from detergent and sterilant. EDTA-based chelating agents added to the detergent help dissolve beerstone and will control accumulation. The level of chelating agent needed can be calculated from the calcium level in the mash. A problem recently encountered is sodium contamination of process condensate water. During CIP the caustic based detergent can be neutralized by the CO_2 in the fermentor producing large quantities of soluble sodium carbonate that can pollute the process condensate. Very high sodium levels can weaken the yeast during fermentation and care must be taken to reduce these sodium levels.

CONTINUOUS FERMENTATION

The most successful continuous fermentation system used in distilling is the cascade system (Figure 9). The system illustrated in Figure 9 has at least two fermentors and a beer well where the yeast are recycled. Yeast can only be recycled when clear mashes are used. The yeast can then be centrifuged, washed and reused. Typically, yeast can be washed using phosphoric acid at a pH of 2.2-2.4 and a holding time of 90 minutes. These conditions are sufficient to kill the bacteria without doing permanent damage to the yeast. Chlorine dioxide at 40-50 ppm is an alternative to acid washing, and seems to be gentler on the yeast.

The system in Figure 9 has two fermentors, whereas most cascade plants have five fermentors and a pre-fermentor. The majority of these plants in the US use whole mash fed into the first two fermentors. Both are aerated continuously with sterile air and are cooled with external coolers. The pre-fermentor feeds yeast equally into both. The mash has been previously saccharified and enters the fermentors at a pH of 4.0 or slightly less. Usually these systems, if yeast stress factors are taken into account, produce 13-15% beers in 24-30 hrs although clear mash systems operate in 10-14 hrs. Each fermentor is individually cooled and agitated either with air or CO_2 or mechanically. The clear mash systems are capable of maintaining a fixed yeast count although they have the potential to recycle.

Typically yeast cell counts in these fermentors are slightly higher than in batch systems, with counts in the range of 180-220 million cells/ml for the first two fermentors and slightly less as the mash proceeds through the system. The viability and percentage of budding cells decrease from one fermentor to the next. Typically, the beer entering the beer well could be down to 120 million cells/ml with a viability of around 30%. Alcohol levels could be 10-11% in Fermentors 1 and 2, 12-13% in Fermentor 3 and up to 15% in Fermentors 4 and 5.

The main advantages of continuous fermentation (and they are very important advantages) are rapid throughput and the fact

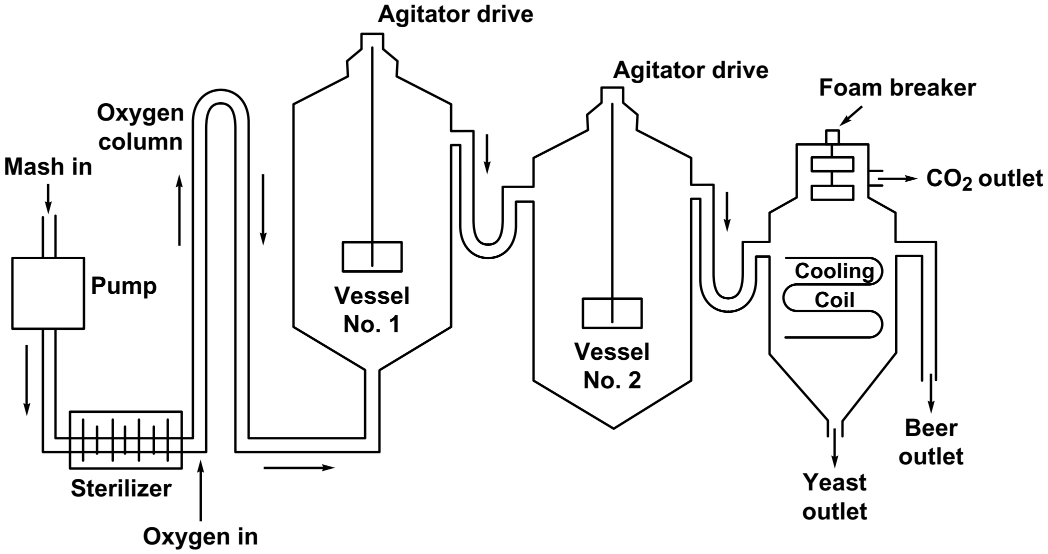


Figure 9. Twin vessel continuous stirred fermentor.

that these fermentors can be run for very long periods without stoppage. Cleaning costs are therefore practically eliminated; and vessel utilization is superior to any batch system. These continuous systems are frequently used for 12 months without stopping and are only cleaned when the plant has its annual shutdown.

The most serious problem encountered with the cascade system is infection. The fermentors can be infected through non-sterile air injected into the first two fermentors. In this case, acetic acid bacteria, which convert the ethanol to acetic acid, can be introduced. This can be detected by a vinegar odor, or more accurately by HPLC analysis. There is also a possibility of infection by lactobacilli that usually propagate in contaminated mash coolers or saccharification tanks.

The new distillers, at least those in the US, have elected not to use continuous fermentation and are instead using the batch process. Batch systems are more easily controlled, more flexible and capable of higher ethanol levels.

Conclusions

Understanding the stress factors that affect yeast and the fermentation equipment will help us move to the theoretical 23% ethanol that yeast such as Thermosacc™ are capable of producing. It is critical, however, that we push the positives: ‘spoon-feed’ sugar to yeast, provide yeast with peptides to ensure high cell numbers in early stages of fermentation, control temperature and overcome the negatives. These negatives, infection, lactic acid, acetic acid, phytic acid and mycotoxins all prevent yeast, and therefore the distillery, from achieving potential yields.

Today distilleries must achieve yields of 2.9 gallons per bushel. Tomorrow’s distilleries will need over 3.2 gallons per bushel. To do this, yeast must remain the central focus of all efforts. Doing so will allow 23% ethanol to soon become the norm.

Chapter 11

Continuous fermentation in the fuel alcohol industry: How does the technology affect yeast?

W.M. INGLEDEW

Applied Microbiology and Food Science Department, University of Saskatchewan, Saskatoon, Saskatchewan, Canada

Introduction

Fuel alcohol manufacture in North America is conducted in approximately 70 (soon to be 78) production plants. The annual output from North American fuel alcohol distilleries will soon exceed 3 billion US gallons (11 billion liters). Both continuous and batch fermentation technologies are used, with the breakdown between plants (Table 1) and on a volume basis (Figure 1) not exactly illustrating the fact that older and larger wet mills predominantly use continuous technology whereas the newer and smaller dry mills predominantly use batch fermentation.

Table 1. Fuel alcohol plants using continuous or batch fermentation (2003).

	Numbers of US plants (70 total)	
Volume	*Continuous*	*Batch*
<11 million gal/year	3	15
12-39 million gal/year	9	15
>40 million gal/year	14	14

In this article, continuous fermentation, the prolonging of the logarithmic phase of yeast

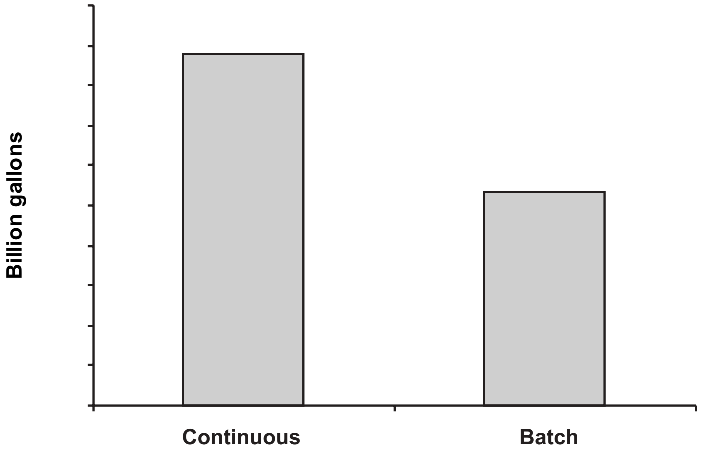


Figure 1. Volumes of alcohol made in 2003 in 70 US plants by batch and continuous technology.

growth seen in batch culture, will be the focus along with a discussion on the technology and information relating to problems that yeast have in such environments.

Continuous fermentation

Continuous fermentation is said to be advantageous because of elimination of the lag phase of yeast growth, the yeast being 'locked' into exponential phase growth where conditions exist for maximal ethanol formation, long term continuous productivity, higher volumetric productivity, reduced labor costs while in steady state, reduced downtime for cleaning, filling and sanitation, easier process control and savings in construction of smaller fermentors with higher output (Cyzewski and Wilkie, 1978; Kirsop, 1982; Kosaric *et al.*, 1987; Maiorella *et al.*, 1981; Sinclair and Cantero, 1990). Higher technological capability to operate such systems is needed, but the primary disadvantages in continuous culture are the problems with contaminating bacteria and wild yeast that disturb the balanced nature of the fermentors, problems of maintenance of high fermentation rates, and genetic stability of the culture used – all of these negating, in part, the advantages mentioned above. Contamination leads to losses in yields of ethanol and unplanned shutdowns of the fermentation trains – factors that lead to lower than expected yields of product and less efficient conversion of sugars (from starch) to ethanol (Bayrock and Ingledew, 2001b). In the fuel ethanol industry, most continuous fermentation is done by the wet milling segment of the industry with the advantage that ethanol production can occur over prolonged periods of time under optimal environmental conditions.

SINGLE STAGE CONTINUOUS FERMENTORS

This most simple form of continuous fermentation is diagrammed in Figure 2. In such a system there is a continued flow (F) of nutrients into the fermentor from the medium reservoir, and a continual flow of product (P) out of a fermentor to a harvest reservoir. The fermentor is designed such that the internal volume of 'medium' remains constant.

The parameter describing this is called the dilution rate (D), measured in hr^{-1}, calculated by

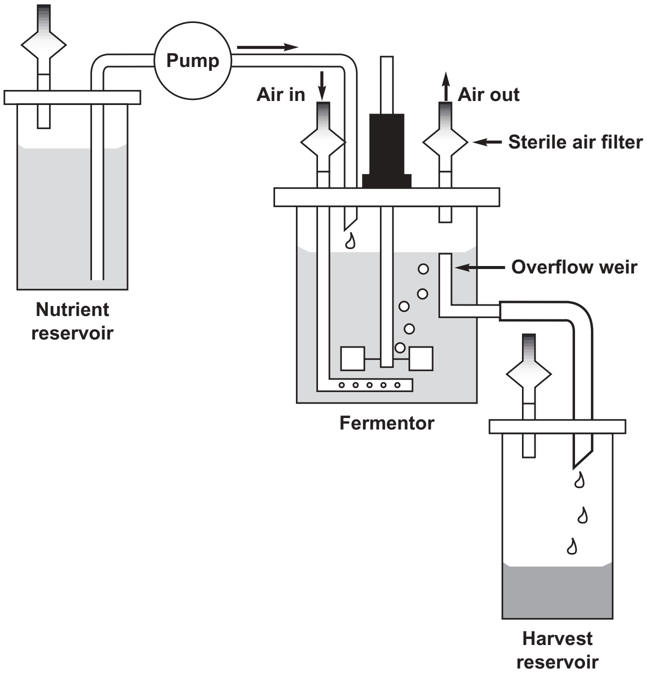


Figure 2. A schematic diagram of a continuous fermentor. The flow rate of medium into the fermentor is controlled, thereby determining the flow of product into the harvesting reservoir (Waites *et al.*, 2001).

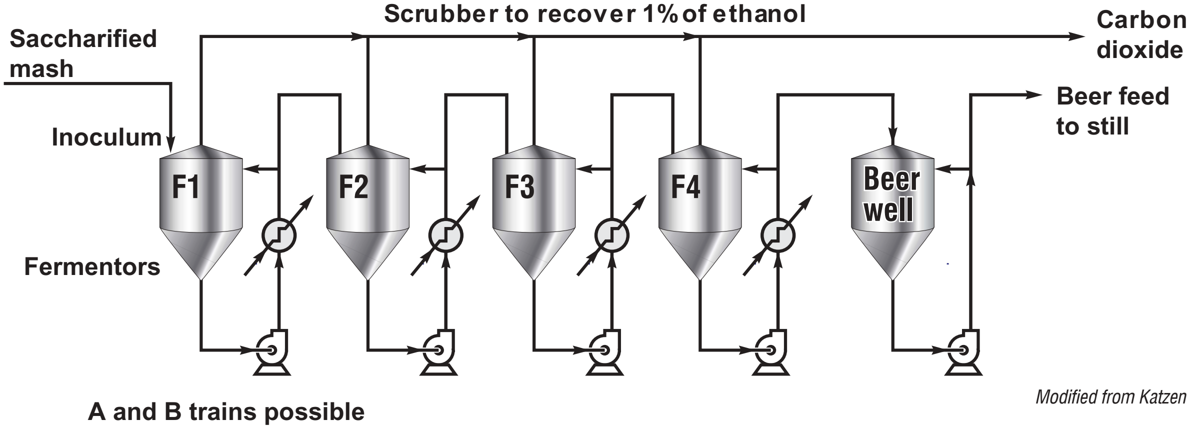
dividing the medium flow rate (F) by the working volume of the vessel (V). The vessel is stirred to ensure continued mixing of the contents. An adequate, constant flow of the same medium ensures that D is constant, and therefore that the yeast cell growth is constant, the substrate level (and product level into the harvest reservoir) are constant. At that time, the vessel will be balanced in a so-called *steady state*. Steady state is governed by the level of a limiting nutrient in the mash medium. It is normally achieved after three residence (replacement) times, where T = 1/D. In some studies, six residence times are used to ensure steady state conditions.

When changes to dilution rate take place, the parameters above (P and S concentrations) as well as the cell biomass all change, and time is needed to bring the vessel back to steady state. The time required depends upon the size of the vessel(s) used and will also depend on any other environmental changes or chemical or biological contamination which can affect the pH, levels of inhibitory compounds present, temperature etc. The rate of cell growth is denoted by μ, the specific growth rate of the yeast. Contaminants have their own specific growth rates (μ) that, in competition with the yeast, may result in faster or slower growth rates than that of the yeast. In such a case, one microbe will wash out of the fermentor, normally leaving the other as the dominant organism. Specific alcohol productivity in a simple continuous fermentor is ordinarily limited by ethanol inhibition and by low cell concentration, necessitating substrate feed concentrations of about 10% w/v (Kosaric *et al.,* 1983).

MULTISTAGE CONTINUOUS CULTURE

In multistage continuous culture in the fuel alcohol industry, four or five fermentors linked in series are normally used. In such a fermentation 'train', each fermentor is usually of the same working volume, and is ideally fed from the one before. The first fermentor is fed by a mash stream that is under continuous production and cooled aseptically to fermentation temperature. Interestingly, if fermentors can be operated in balanced growth, there is no need for added yeast, no need for nutrient supplements or mash additions to the second to fifth fermentors and little need for anything but monitoring. In such cases, each fermentor is in balanced growth where the substrate level is constant, the product level is constant, the rate of fermentation is constant and so is the amount of yeast biomass (viable yeast) that, of course, is the catalyst of the biochemical reaction. However, none of the fermentors in the train have the same steady state values as fermentors upstream or downstream. Dilution rates, however, will be identical if the volumes contained are the same and if no additional mash or other additions are made distal of Fermentor 1. Productivity on continuous processes can exceed 6 g ethanol/L fermentation mash/hour (>2.4 to 3.3-fold more than corresponding batch fermentation – Kosaric *et al*., 1987), and this means that for similar plant outputs, vessels can be 3-fold smaller, and operating and capital costs are reduced by ~50%.

Figure 3 shows a simple engineering diagram of a continuous train. This is a train with four



Modified from Katzen

Figure 3. A continuous multistage fermentation train as designed by Katzen International Inc.

fermentors and a beer well. The inoculum is added when needed, and saccharified mash is continuously provided to Fermentor 1. Cooling is provided to each fermentor and an effluent takeoff is sent to the second fermentor at the same rate of addition as that of the mash entering Fermentor 1. Carbon dioxide leaves each fermentor, and it is scrubbed to remove ethanol (in this case about 1%) and any dissolved solubles or entrained particulates. Often in such designs, two identical trains are built. This gives a remarkable opportunity for any changes made in the A train to be compared to the unchanged B train – during and after a period of equilibration to steady state.

Figure 4 is a photograph of a typically installed multistage continuous culture. This fermentation train has been a model of steady state fermentation where for long periods of time, balanced growth was maintained, and the fermentor during this time did not require additional yeast cells to be added.

There are many possible variations in a designed multistage continuous fermentation. Some involve a pre-fermentor, a yeast propagator, or additions of nutrient mash streams into more than one fermentor. In all such schemes, balanced S, P, or biomass is more difficult to maintain in any vessels distal to the last one where nutrients are added or where changes occur.

Another advantage in operation of a multistage train is that the first vessel can be used to produce cells (viable biomass) that will be used to transform remaining substrate in Fermentors 2 to 4 to end product. For example, if the volume of Fermentor 1 was twice the volume of the other production fermentors, the dilution rate would be lower (and average medium residence time would be higher) leading to more extensive cell growth. Conditions would be imposed on Fermentor 1 to ensure maximum growth, while conditions in Fermentors 2 to 4 (or 5) could be more optimal for ethanol production. If a medium feed occurs in Fermentor 2 as well as in Fermentor 1 (called distributed feeding), the mathematics at steady state for substrate utilization, product formation, dilution rate, etc. will be different and more complex. A mathematical treatment of continuous fermentation can be found in the PhD thesis of D. Bayrock (2002).

CONTINUOUS CULTURE WITH YEAST RECYCLE

Figure 5 portrays a continuous culture system with provision for yeast recycle. This is the so-called Biostil fermentor system where effluent from the continuous fermentor is centrifuged, and the collected yeasts are re-added to the

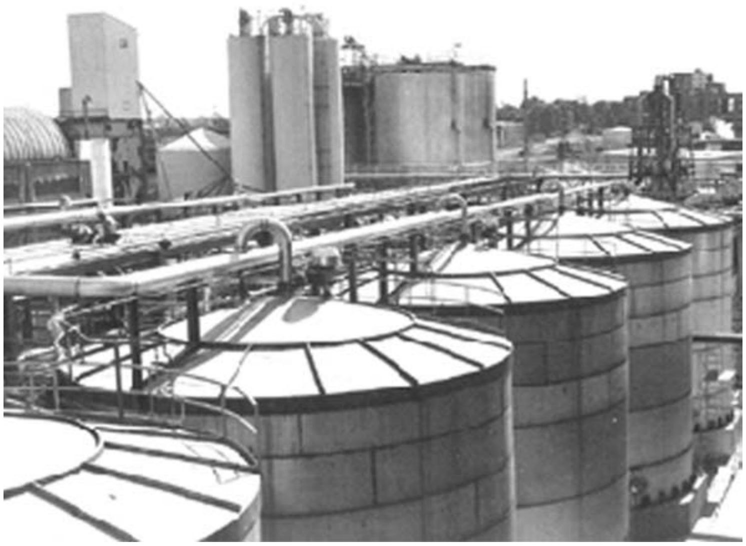


Figure 4. The continuous fermentation train previously operated by Williams Energy in Peoria, IL.

fermentor to provide additional catalytic activity and therefore faster production of ethanol (Danielsson, 1992). The advantages of this design include the continuous removal of yeast by centrifuge, an increase in yeast cell density in the fermentor of 6 to 10-fold, and a lesser synthesis of new yeast with the concomitant small increase in sugar available for ethanol production. Unfortunately, the viability of recycled Biostil yeast is not good. Solids accumulate with the yeast and build up due to recycle (the reason why the design is more of importance in sugarcane juice fermentation than in corn mash fermentations), bacteria and wild yeast increase in number and lead to accumulation of inhibitory metabolites, and centrifuge/separation is required. Operating costs and capital expenditures increase due to the need for cell separators and reinoculation. The literature reports ethanol productivities as high as 50 g ethanol/L/hour in these fermentation systems – directly due to increased yeast biomass. Yeast is the catalyst in the reaction converting sugar to ethanol, carbon dioxide and glycerol. It is said that control of infection in such a system takes place due to the low pH, the low content of fermentable sugar (usually less than 0.1%), the high osmotic pressure, the yeast concentration of up to 10^9 (1000 million) cells/mL, the 6-9% v/v ethanol present, and the fact that internal pasteurization using a heat exchanger can be accomplished. A one vessel system is also easier to shut down, sanitize and restart.

Continuous cultures, regardless of design, must operate simply and at maximum capacity and maximum efficiency. They must produce ethanol as well as by-products of consistent quality. As said previously, the multistage systems in use in fuel alcohol production are often somewhat complex in that more than one fermentor is fed, a pre-fermentor or yeast propagator is often engineered into the system, and the presence of continued infection leads to abnormal stresses that take the fermentors out of balanced growth. Reestablishment of balanced growth can take well over 48 hrs, and this means that the plant is continually destabilized and that the lab and plant staff are continually challenged to find the key to stabilize conditions.

It is especially a challenge to reduce or eliminate bacteria without losing productivity (ethanol made per working volume per hr). It is normally not possible to clean and sanitize one fermentor without later cross-contaminating it by the others in the train. This is because all are

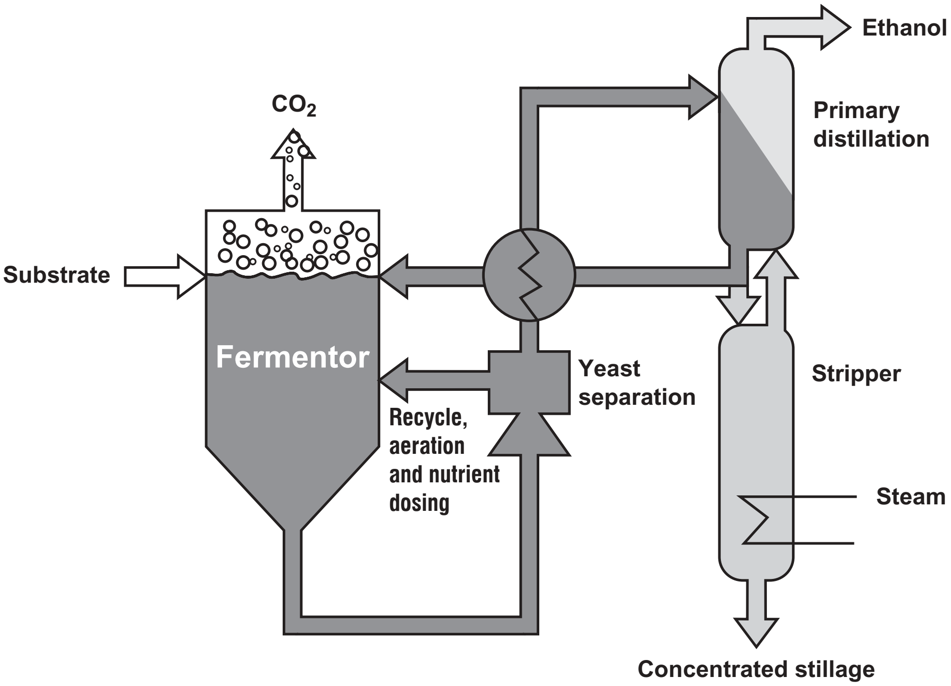


Figure 5. The Biostil fermentor system (Chematur Engineering, Concept of Continuous Alcohol Production, 1993).

contaminated in the flow of the liquid through the train, and when Fermentor 1 is taken down for cleaning and then restarted, it quickly becomes contaminated from the CO_2 headers that often connect the train in a countercurrent direction. Headers are also very difficult to clean. In addition, the mathematics and engineering theory are so complex in comparison to batch fermentation that it is not easy to obtain adequate advice on eradication of problems. Moreover, plants often make changes to the parameters under which the train operates, and do not allow enough time to see if the train re-establishes productivity (steady state) before even more changes are made.

Another problem inherent in the operation of continuous multistage systems is that saccharification of starch must be carried out at the optimal temperature for the enzyme prior to addition of the mash medium to Fermentor 1. The saccharification stage is ideally suited for growth of very heat tolerant *Lactobacillus* strains known to create the lactic acid in fuel alcohol plants that interferes with yeast metabolism. Simultaneous saccharification and fermentation (SSF) technologies were developed for batch fermentation to eliminate this very important source of bacteria and the resultant loss of ethanol yields. SSF is not possible in continuous systems at this time. Moreover, in a multistage train, saccharification, yeast propagation, pre-fermentation, and fermentation are the normal sequence of events. This complicates the total understanding of changes that occur in the train with time. As the section below and the chapter in this volume on water usage show, balanced growth is very susceptible to wastewater streams and surges of such wastes (such as sodium hydroxide (NaOH) in wash water, evaporator condensate, thin stillage, still bottoms, or biomethanator discharge) and this, compounded by changes in mash composition, temperature, osmotic pressure, and bioproducts of contaminants including acetic acid and lactic acid all can lead to perturbations of the train that are difficult to repair. In addition, our lab has shown that deficiencies in yeast-usable nitrogen occur in mashes made from every conceivable grain substrate. Such constituents vary from load to load of received grain, making continuous operation difficult due to the fact that yeast levels in mash vary with nutrition. Even small changes in dilution rate can lead to washout of yeast (insufficient time to multiply), which eventually leads to lowered productivity.

It is also the belief of this author that continuous systems may never be able to produce ethanol at levels over 15-16% v/v, due to washout of cells occurring because the yeasts are continually bathed in ethanol and because the levels of other medium ingredients (some recycled in backset) also increase providing continual, synergistic stresses that lower metabolic rates and decrease ethanol levels during the residence time of the fermentors. As substrate levels are increased (concomitantly with alcohol concentrations), the dilution rates become smaller such that for all practical purposes, the fermentors approach batch mode rather than remaining in a continuous mode.

It should be mentioned from an academic standpoint that other continuous systems have been developed, including: internal recycle (flocculation of cells), tower fermentors, membrane reactors, vacuum fermentation (removing ethanol and carbon dioxide), solvent extraction (removing ethanol), and immobilized cell reactors. None of these have had much importance in manufacturing ethanol except in the brewing industry.

Contamination and stressful conditions in a fermentation plant

The source of microbes in distilleries is not mash prior to its cooling, due to the high temperatures used for starch gelatinization and liquefaction. All points in the alcohol process after cooking, however, can be foci for infection including coolers or heat exchangers, tankage, transfer lines, unprocessed waters, yeast, enzymes and other additives, air, fermentor tankage or the beer well. Viable contaminants and their end products in recycled waters can all affect subsequent fermentations. Contaminants of concern to fuel alcohol plants mainly include the *Lactobacillus* bacteria and wild yeasts. It is important that lactobacilli do not grow well in corn mash and are not able to grow at low pH. However, some are well adapted and grow exceedingly quickly under mash fermentation conditions. For the purpose of this chapter, it is important to realize that bacteria and wild yeast compete with *Saccharomyces* yeast for nutrients. Moreover, and more importantly, the end products of the metabolism of the bacteria are normally lactic acid and smaller amounts of acetic acid, whereas the wild yeasts (like *Brettanomyces)* produce acetic acid (in addition to ethanol and carbon dioxide).

It is these contaminant end products that cycle throughout the plant due to the fact that they are not as volatile as ethanol and mostly remain in the thin stillage. For this reason, waste waters such as backset and evaporator condensate are critical components in mash manufacture because they contribute increasing amounts of chemicals that inhibit yeast growth, and the levels of such chemicals increase with every subsequent cycle. Many, if not most, problems in fermentation can be alleviated if a plant can eliminate backset and/or evaporator condensate, replacing them in the mash bill with water for one or more cycles. This emphasizes the need for excess evaporator capacity in newly designed facilities.

CONTAMINATION OF ACTIVE DRY YEAST

This subject of entrance of bacteria into mash via pipes, unclean tanks, or air, etc. is covered in the chapter by Larson and Power in this volume and found in books discussing cleaning and sanitation. A word is needed, however, on levels of contamination in both active dry yeast and via plant-propagated yeast in order to understand how fermentations can become contaminated in-house.

Yeast can be a source of contamination. Active dry yeasts (ADY) cannot be grown, harvested and dried without a level of bacterial, mold and wild yeast contamination. Usual levels are given in Table 2.

Table 2. Normal contamination in active dry yeast (organisms/g).

Bacteria	*Wild yeasts and molds*	*Culture yeasts*
10^3-10^5	<10^2 - 10^3	2.2 to 5 × 10^{10}

Considering the levels of contaminants, and usual levels of inoculation of active dry yeasts into fermentors (2.2 lb/1000 gal US), it can be estimated that the contribution of the bacteria in the mash after a one-time inoculation of ADY is less than 26 viable bacteria per ml of inoculated mash. That of the wild yeast and molds would approximate 2 cells per ml. At such levels, it appears obvious that the bacteria would need to have a very definite competitive advantage in the fermentor (or a prolonged time) before significant interference in the process would occur. This is why a batch fermentation process has little worry about contaminants in ADY. A continuous fermentation train could be in difficulty using direct inoculation of ADY if the bacteria, wild yeast or mold present in the ADY grew faster in the mash provided under the environmental and chemical conditions (and time of operation) of the fermentation train. In all likelihood the contaminants from equipment would exceed these numbers by a considerable amount. The lowering of pH practiced in plants suffering contamination often lowers the growth rate of the bacteria present such that they wash out of the fermentation, leaving the more pH tolerant yeast to multiply and persist. However, the cost of this procedure is lowered alcohol yield.

Yeast repropagation in a fuel alcohol plant is a different story especially when continuous propagation is practiced. Under such conditions, the very small number of bacteria mentioned above that are present with the yeast in commercial ADY preparations are provided an opportunity to grow and compete. Because bacteria normally have significantly shorter generation times (times required to double), those few bacteria that can endure the conditions of the fermentor (like Lactobacillus) will compete with *Saccharomyces* yeasts for nutrients and eventually increase in numbers to the extent that they begin to produce large amounts of lactic acid and some acetic acid which inhibit yeast and help them to take over the population in the propagator, and in similar fashion, the fermentors. These end products are known to affect the growth of culture yeast and reduce alcohol output in fermentors when these vessels become contaminated at levels over 105 bacteria (100,000) per mL (Narendranath *et al*., 1997). As acids build up to over 0.1% w/v (lactic acid) and 0.05% w/v (acetic acid), we know that the length of the yeasts' lag time after inoculation will increase significantly (Figure 6) as does yeast generation time (Narendranath and Ingledew, 2001a). For these reasons, continuous propagation is not recommended by this author.

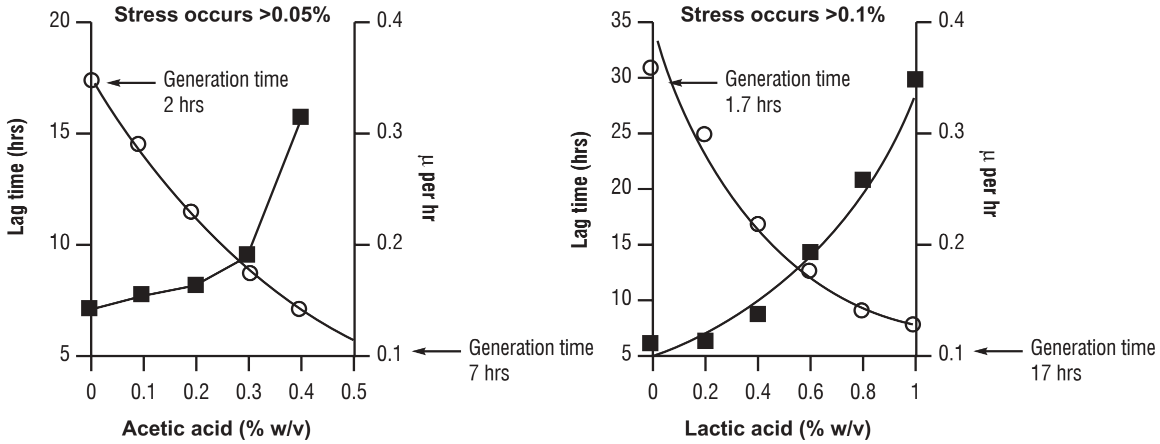


Figure 6. Specific growth rates decrease and lag times increase exponentially as acids increase in concentration. MIC (acetic) = 0.6%, MIC (lactic) = 2.5%. MIC = no growth at 72 hrs. ○ = specific growth rate, ■ = lag times; generation time = ln2/μ.

CONTAMINATION CONTROL STRATEGIES

The effects of bacterial end products when more than one stress is present are additive, or more likely synergistic, and affect the yeast in association with other stressful situations like high ethanol, high temperature and low pH. The yeasts are then in serious trouble (Narendranath *et al.,* 2001a). The mechanism of action of the stressful chemicals is now better known (Narendranath *et al.,* 2001b). For this reason, it is necessary to reduce the numbers of the microbes present in mash such that the levels of bacterial end products are too small to affect rates of ethanol production or ethanol yields. This is normally done with low pH, antimicrobials or antibiotics.

The use of antibiotics in continuous culture has been more of an art than a science. Unfortunately, in the past most applications of antibiotics have been done with the same thought process that had been in use for batch fermentations. Antibiotics were, therefore, added all at once into continuous systems with the result that a spike of antibiotic concentration took place, which decreased over time at a rate prescribed by the set dilution rate (D). In 2001, the published work by Bayrock and Ingledew (2001a; 2001b) provided alternative strategies for the provision of antibiotic to continuous systems. This work was done in a bench scale multistage continuous culture system, which was designed to model the commercial fermentation train at Williams Energy in Pekin, IL. The work was conducted with penicillin G, an antibiotic long used in the industry. Luckily, penicillin is not degraded enzymatically by *Saccharomyces* yeasts. The penicillin was therefore added in pulses considering scientifically only its degradation rate at the pH of the mash, its degradation rate at the temperatures used over the time course of the continuous system, and the dilution rate of the fermentor (and each component in it). It was known that in the system used only 49.8% of the antibiotic would remain after 3.2 hrs of incubation in mash at pH 6 at 28°C, with only ~1% remaining at 21 hrs. The regime for addition therefore included antibiotic pulses every 6 hrs with an average penicillin concentration of 2475 U/liter but with spikes of larger values than the average such that bacteria would be alternatively inhibited from growing - and perhaps also encouraged to initiate growth again when concentrations were lowest. This was found to be more effective than the continuous addition of the same antibiotic over time to the same average value (Bayrock and Ingledew, 2003). This technology is being translated to industrial use. Other single antibiotics are available to the industry as are commercial formulations with combinations of antibiotics providing a broader target range against contaminating bacteria. The latter reduce a number of persistent infections that detract from ethanol yield.

Conclusions

The economic impact of the presence of contaminating bacteria in fermentation mashes is profound. When bacterial numbers reach 10^6 (one million) to 10^7 (10 million) per mL, losses in alcohol of 1-3% can be seen (Dolan, 1979; Narendranath and Ingledew, 2001a). A 3% loss in a 19.3 million gal/year (53,000 gal/day) alcohol plant is a loss of 580,000 gallons of ethanol per year! These very real economic considerations dictate that bacteria and fast-growing wild yeasts must be controlled in continuous fermentation.

References

Bayrock, D.P. 2002. Application of multistage continuous culture to VHG-based ethanol fermentations: performance and control of bacteria by pH and pulsed addtion of antibiotic. PhD Thesis. University of Saskatchewan. Saskatoon, SK Canada.

Bayrock, D. and W.M. Ingledew. 2001a. Changes in steady state on introduction of a *Lactobacillus* contaminant to a continuous culture ethanol fermentation. J. Ind. Microbiol. Biotech. 27:39-45.

Bayrock, D. and W.M. Ingledew. 2001b. Application of multistage continuous fermentation for production of fuel alcohol by very-high-gravity fermentation technology. J. Ind. Microbiol. Biotech. 27:87-93.

Bayrock, D., K.C. Thomas and W.M. Ingledew. 2003. Control of *Lactobacillus* contaminants in continuous fuel ethanol fermentations by constant or pulsed addition of penicillin G. Appl. Microbiol. Biotech. (in press). On line May 13, 2003.

Cysewski G.R. and C.W. Wilkie. 1978. Process design and economic studies of alternative fermentation methods for the production of ethanol. Biotech. Bioeng. 20:1421-1430.

Danielsson, M.A. 1992. Continuous fermentation of concentrated feed stocks in a single fermentor. Fuel Alcohol Workshop, Wichita, KS.

Dolan, T.C.S. 1979. Bacteria in whisky production. The Brewer. 65:60-64.

Kirsop, B. 1982. Developments in beer fermentation. Topics Enz. Ferment. Biotechnol. 6:79-131.

Kosaric, N., A. Wieczorek, C.P. Cosentino and R.J. Magee. 1983. Ethanol fermentation. In: *Biotechnology Vol. 3*. (H.-J. Rehm and G. Reed, eds). Verlag Chemie, Weinheim. pp 257-385.

Kosaric, N., Z. Duvnjak, A. Farkas, H. Sahm, O. Goebel and D. Mayer. 1987. Ethanol. In: *Ullmann's Encyclopedia of Industrial Chemistry*. 5th Edition. Vol A9. (W. Gerhartz, ed), VCH Publishers, N.Y. pp 587-653.

Maiorella, B., C.R. Wilkie, and H.W. Blanch. 1981. Alcohol production and recovery. Adv. Biochem. Eng. 20:13-92.

Narendranath, N.V., S.H. Hynes, K.C. Thomas and W. M. Ingledew. 1997. Effects of lactobacilli on yeast-catalyzed ethanol fermentations. Appl. Environ. Microbiol. 63:4158-4163.

Narendranath, N.V., K.C. Thomas and W.M. Ingledew. 2001a. Effects of acetic acid and lactic acid on the growth of *Saccharomyces cerevisiae* in a minimal medium. J. Indust. Microbiol. & Biotech. 26:171-177.

Narendranath, N.V., K.C. Thomas and W.M. Ingledew. 2001b. Acetic and lactic acid inhibition of growth of *Saccharomyces cerevisiae* by different mechanisms. J. Am. Soc. Brew. Chem. 59:187-194.

Sinclair, C.G. and D. Cantero. 1990. Fermentation modeling. In: *Fermentation: A Practical Approach*. (B. McNeil and L.M. Harvey, eds). Oxford University Press, London UK. pp. 65-112.

Waites, M.J., N.L. Morgan, J.S. Rockey and G. Higton. 2001. Industrial Microbiology: an Introduction. Blackwell Science Ltd. Oxford, UK.

Chapter 12

Understanding near infrared spectroscopy and its applications in the distillery

DON LIVERMORE[1], QIAN WANG[2] AND RICHARD S. JACKSON[3]

[1]*Research Scientist, Hiram Walker & Sons Ltd., Walkerville, Ontario, Canada*
[2]*NIR Product Manager, Bruker Optics Inc., Billerica, Massachusetts, USA*
[3]*Applications Manager, Bruker Optics Inc., Billerica, Massachusetts, USA*

Introduction

Near-infrared (NIR) spectroscopy has been a reliable technique in the Hiram Walker & Sons distillery quality assurance laboratory since 1997. It has proven to be powerful tool that can benefit distilleries, breweries, or other producers of other types of ethanol in quality assurance programs and research facilities. As a research tool NIR spectroscopy can be used to evaluate raw materials, yeasts, enzymes, nutritional supplements, and production parameters to optimize conditions for a production plant. As a quality assurance tool it can assist production by monitoring and maintaining control of processes. If failures are not quickly identified and a process becomes out of control, it can cost valuable time, product, and resources. NIR spectroscopy can help eliminate these problems.

What makes NIR technology so attractive to the ethanol industry as a quality assurance and research tool is that it can measure organic substances very quickly (5-10 seconds) and consistently without the destruction of a sample. Traditional analysis can take a lab technician several hours or even days to get reliable results, but with NIR spectroscopy the result is virtually instantaneous and does not use hazardous chemicals. The areas of production where rapid and reliable analysis are most beneficial include monitoring of incoming grains, profiling fermentors, distillate analysis, dry house operations, and finished product analysis. The exact locations where NIR spectroscopy is used in the Hiram Walker & Sons distillery, and the advantages and disadvantages of the technology, will be discussed in more detail. Initially background material for NIR spectroscopy will be discussed, to help the reader understand what it takes to implement NIR spectroscopy in a quality assurance or research program.

History of NIR spectroscopy

Infrared spectroscopy has been a well-established research tool in academia and in industry for several decades. The first experiments conducted with NIR light were by Herschel in 1800, in which he used a glass prism to demonstrate that there was radiation beyond visible light (Herschel, 1800). NIR spectra are complex however, and only recently have computers become powerful enough to analyze the spectra of complex samples. NIR technology is now also a choice for process monitoring equipment, because there is no sample preparation required and fiber optics can be used to transmit the light over significant distances. This has allowed companies in the food and beverage industry such as ethanol producers, to move from low technology to high technology production methods.

NIR background

A detailed discussion of the theory of NIR spectroscopy is beyond the scope of this chapter, but a review of the basics may help the reader understand why NIR is a powerful tool in industry today.

Light consists of electromagnetic waves of varying wavelengths (Figure 1). Our eyes can only sense light at particular wavelengths, called the visible spectrum, but the electromagnetic spectrum covers a much wider range. The near-infrared region of the spectrum extends from 800 to 2500 nm (12500 to 4000 cm^{-1}), lying between the mid-infrared (MIR) region at longer wavelengths and the visible region (VIS) at shorter wavelengths.

When light impinges on matter, each wavelength present will be selectively absorbed, scattered, reflected, or transmitted. *Absorption* is the transition of a molecule from a lower energy level to a higher energy level with the transfer of energy from the light to the molecule. *Scattering* is the redirection of light due to the interaction with matter. Scattering may or may not occur with a transfer of energy and the wavelength of the scattered light may or may not be the same wavelength as that of the incident light. *Reflected* light is similar to scattered light, but the wavelength of reflected light is always the same as that of the incident light. Scattering and reflection of a particle are dependent on the relation between the wavelength of the light and the dimensions and orientation of the particle. *Transmitted* light is non-redirected light, which if it interacts with matter maintains the same wavelength after interaction.

Absorption of infrared light by a molecule is due to interaction of the electromagnetic radiation with the vibration of bonds between atoms. To absorb infrared light there must be a change in dipole moment during the vibration, so homonuclear diatomic molecules such as H_2, O_2, and N_2 cannot be monitored using infrared spectroscopy. In the mid-infrared light is absorbed if the frequency of the light is the same as the fundamental frequency of vibration of the molecular bond. Molecular vibrations are slightly anharmonic, however, and consequently higher frequency light in the NIR can also be absorbed if its frequency is the same as that of one of the harmonics of the fundamental. It is also possible that vibrations can interact and couple, if the vibrating bonds are joined to a single, central atom. The absorption bands in the NIR are thus referred to as either overtone bands, with frequencies of 2, 3 or more times the fundamental, or combination bands with

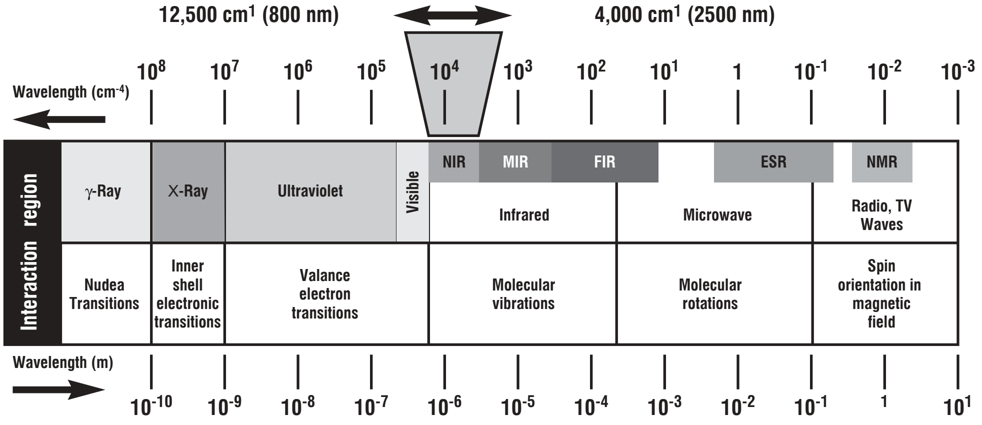

12,500 cm1 (800 nm)
4,000 cm1 (2500 nm)
Wavelength (cm-4)
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
1
10^-1
10^-2
10^-3
Interaction region
γ-Ray
X-Ray
Ultraviolet
Visible
NIR
MIR
FIR
Infrared
ESR
Microwave
NMR
Radio, TV Waves
Nudea Transitions
Inner shell electronic transitions
Valance electron transitions
Molecular vibrations
Molecular rotations
Spin orientation in magnetic field
Wavelength (m)
10^-10
10^-9
10^-8
10^-7
10^-6
10^-5
10^-4
10^-3
10^-2
10^-1
1
10^1

Figure 1. Location of the NIR region of the electromagnetic spectrum in relation to other wavelengths.

frequencies that are the sum of the frequencies of two fundamentals. The NIR absorption mechanisms are much weaker that those in the mid-infrared, and therefore the absorption bands are also much weaker. The strength of the absorption will decrease by a factor of 10–100 for each step from the fundamental to the next overtone. Typical pathlengths (the distance that the light travels through the material) in the NIR are therefore 1-10 mm, compared with 10-100 µm in the mid-infrared.

For overtone or combination bands to occur in the NIR, the frequencies of the fundamental vibrations must be high enough, otherwise these bands occur at wavelengths in the mid-infrared. This is only true for bonds involving hydrogen, for example C-H, O-H, N-H, and S-H. The exception to this is the carbonyl vibration, C=O, which is strong enough in the mid-infrared that the second overtone can be seen in the NIR.

The absorption of light as a function of wavenumber (or wavelength) is known as a *spectrum*. An infrared spectrum typically contains a large number of peaks, which in the NIR are typically broad and overlapped. Example spectra of water and ethanol are shown in Figure 2. The y-axis indicates absorbance, which is the negative log of the fraction of light transmitted by the sample.

NIR instrumentation

TYPES OF NIR SPECTROMETERS

To successfully implement NIR spectroscopy it is essential to have the right instrument. The most important factors will depend on the intended use for the instrument, but major ones that should be considered are the flexibility of the instrument when changing from liquid to solid analysis, the user interface and software provided, the sampling method, the cost of the instrument, instrument specifications, and support from the manufacturer.

Filter-based photometers

There are a number of different types of NIR spectrometer. The simplest and cheapest NIR instruments are filter photometers. Photometers are distinguished from the other types of spectrometer that will be considered, which are all spectrophotometers, because they do not produce a spectrum as shown in Figure 2. A photometer uses filters mounted in a rotating wheel to select small ranges of wavelengths in the spectrum. The filter wavelengths are chosen depending on the desired analysis, for example protein, moisture, and oil. Figure 3 shows how a photometer works.

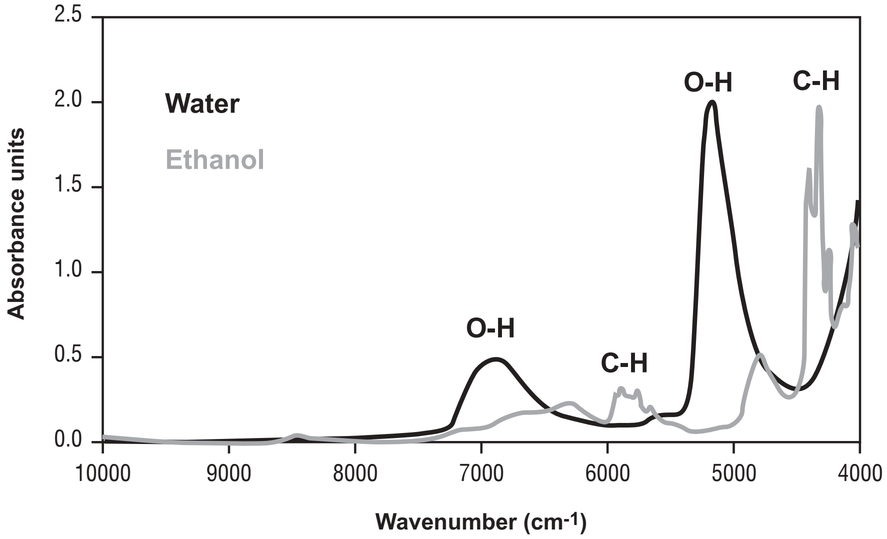


Figure 2. Spectra of water and pure ethanol in the NIR.

Filter photometers lack the flexibility of other instrument types, and can be prone to errors if the temperature changes. They have the advantage of low cost, however, and can be useful for dedicated analysis either in the laboratory or on-line in a production facility.

Scanning dispersive spectrophotometers

In this type of spectrometer broadband light is directed to the sample, and the transmitted or reflected light is then passed through a narrow slit. A diffraction grating then disperses the light into separate wavelengths, which are scanned across an exit slit by moving the grating. The discrete wavelengths that pass through the exit slit are sequentially measured by the detector. Figure 4 shows the principle of operation of a scanning dispersive spectrometer.

Like the other types of spectrophotometer, these instruments are flexible and offer a full range of analytical techniques. Some high-end instruments with double beams are also capable of very high photometric precision. The major disadvantage is that the precision and accuracy of the wavelength calibration are limited by mechanical precision. This can affect instrument stability, existing calibration models, and make transfer of a calibration model to a new instrument difficult.

Detector array dispersive spectrophotometers

In a detector array dispersive spectrometer the scanning grating, exit slit, and detector in Figure 4 are replaced by a stationary grating and a detector array. Since different wavelengths fall on different detector elements they are measured almost simultaneously, so detector array dispersive spectrometers can be very fast. The limited number of elements in the array, however, means that this type of spectrometer is usually either low resolution, or only covers a limited portion of the spectrum. With no moving parts, these instruments are also very rugged.

Acousto-optical tunable filter (AOTF) spectrophotometers

In an AOTF spectrometer the grating in Figure 4 is replaced with an acousto-optic filter. This is a crystal that acts as a diffraction grating when

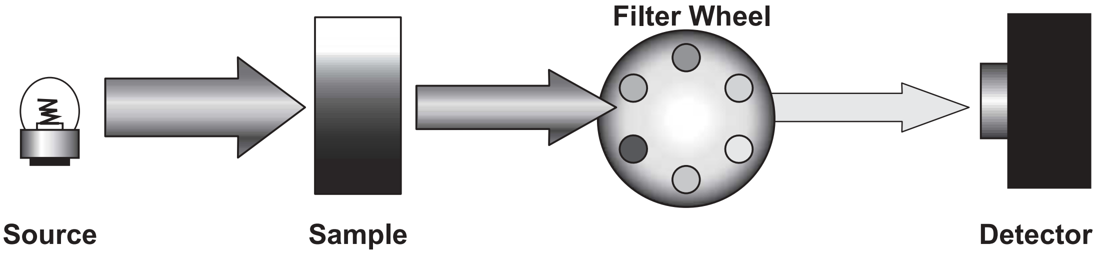


Figure 3. Schematic representation of filter photometer operation.

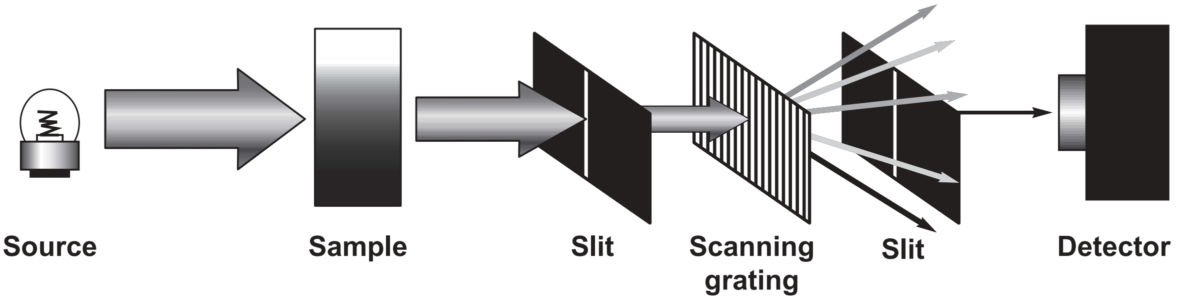


Figure 4. Schematic representation of the principles of a scanning dispersive spectrophotometer.

excited by sound waves using a piezo-electric transducer. The grating can be tuned to different wavelengths by changing the frequency of the sound. These instruments are fast and rugged, but have limited resolution and signal-to-noise ratio. They are also very temperature sensitive, and therefore typically have built-in thermostatting.

Fourier transform (FT) NIR spectrophotometers

A schematic representation of a Fourier transform instrument is shown in Figure 5. The broadband light source is directed to a Michelson interferometer. The interferometer consists of a beamsplitter, a fixed mirror, and a mirror that moves back and forth very precisely. The beamsplitter reflects half of the light to the fixed mirror, and transmits the other half to the moving mirror. Both the fixed and moving mirror will direct the light back to the beamsplitter, where they interfere. This interference changes as the moving mirror is displaced, and therefore the intensity of the light at the detector changes. The intensity as a function of mirror displacement is called an interferogram, which must then be Fourier transformed to obtain the spectrum. A detailed description of the operation of Fourier transform spectrometers can be found in Griffiths *et al.* (1986).

Fourier transform spectrometers offer high resolution, good speed, and high signal-to-noise ratios. Their biggest advantage, however, stems from the fact that the position of the moving mirror is controlled using a HeNe laser. The inherent wavelength stability of the laser results in very high wavelength accuracy and precision. This in turn means a calibration model is very stable over time, and permits easy transfer of calibration models between instruments.

FT-NIR has been the instrument of choice in the Hiram Walker & Sons laboratory because of these advantages, especially the wavelength accuracy and precision. In the alcohol industry measuring spirit strength is very important to regulatory agencies. If NIR were to be approved by governments as an official method of alcohol determination, then the wavelength stability of FT-NIR systems might be advantageous because of the resulting model stability. Currently available systems also offer a full range of analytical techniques (i.e. solids, pastes, powders, liquids, probes, vial holders, integrating spheres) on a single instrument, with easy computer controlled switching between different modes.

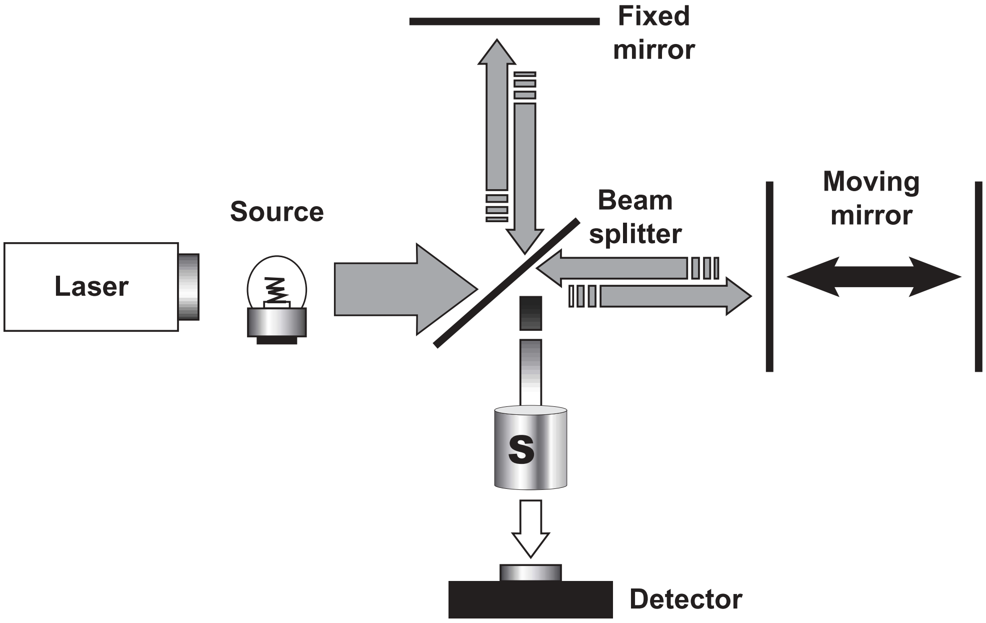


Figure 5. Schematic representation of an interferometer in a FT-NIR system.

Sample presentation

There is a wide range of possibilities in the alcohol industry for NIR analysis. There are three main categories of analysis. The first of these is off-line measurement, where sample materials are selected from production to be analyzed by an instrument in a laboratory setting. This type of sampling places the least demand on the operator developing the calibration models. The second category is at-line sampling, where the instrument is placed close to the operation in order to decrease analysis time. Depending on the environment, the instrument may need to be in an enclosure. Finally, there are sampling accessories that can be integrated into the production line without having to collect samples by hand. This is called in-line analysis. This type of analysis allows the user to integrate the spectrophotometer with existing control systems. In each of these categories there are a number of sampling accessories available, which must be carefully chosen. There are three types of measuring techniques that the accessories can use: transmission, diffuse reflection, and transflection.

TRANSMISSION

In transmission measurements light is directed at a sample, where some of the light is absorbed and some is transmitted to the detector. This type of measurement is used for samples that are liquids or transparent foils (Figure 6).

The main accessories used for transmission measurements are a vial holder or a liquid probe. The benefits of vial holders include a temperature-regulated block, and custom-sized disposable glass vials. The fiber optic-based liquid probe can be used for either off-line, at-line, or in-line measurements. Light from the spectrophotometer is directed through fiber optic cable to a pair of mirrors that direct the light through a cavity in the probe head. The cavity has a fixed pathlength that enables a liquid sample to enter (Figure 7). Figure 8 shows a commercial probe.

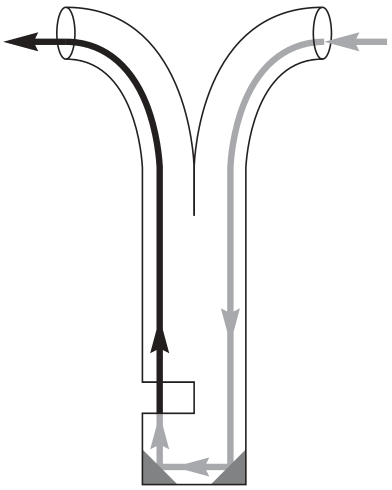


Figure 7. Schematic diagram of a liquid probe, showing the path of the light.

Figure 8. A commercial hand-held liquid probe for off-line or at-line analysis.

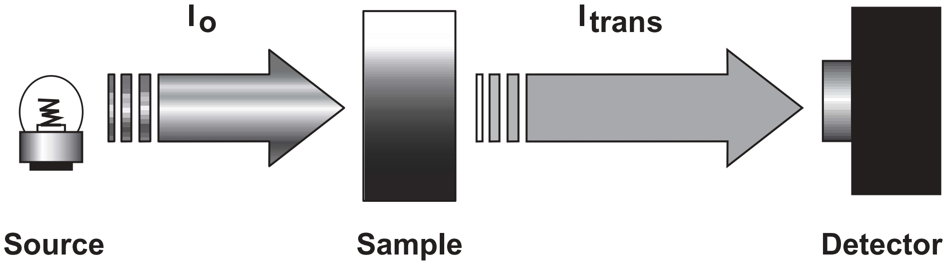


Figure 6. Schematic representation of a transmission measurement.

DIFFUSE REFLECTION

When light is reflected from rough surfaces or powders, it is referred to as diffuse reflection. Depending on the sample, light may penetrate beyond the surface a significant distance. There is therefore the potential of quantifying the components within the sample. There are two types of accessories that measure by diffuse reflection: the diffuse reflectance probe, or the integrating sphere.

The diffuse reflectance probe uses a fiber-optic cable with multiple fibers. Half of the fibers in the bundle are typically used to transmit the light to the sample, and half to return the reflected light to the spectrometer. A schematic diagram of a diffuse reflectance probe is shown in Figure 9.

A second type of diffuse reflection accessory is an integrating sphere. In an integrating sphere light is directed onto a sample, as shown in Figure 10. The reflected light is measured by a detector, which is mounted in the wall of the gold-plated sphere. Typical samples measured by the integrating sphere are distillers dried grains with solubles (DDGS), whole kernel corn, and corn mash. An integrating sphere is well suited to these types of inhomogeneous sample because of the large sampling area. If the sample is very inhomogeneous, as is the case with corn and DDGS, a rotating cup, as shown in Figure 11, can be placed on the integrating sphere to provide further averaging. In the case of corn mash, an electrically powered mixer is needed to stir the sample so the insolubles will not fall to the bottom.

TRANSFLECTION

Transflection is an extension of the reflection technique, as shown in Figure 12. When a mirror is placed behind the sample, the light transmitted through the sample is reflected back through the sample and into the diffuse reflectance probe or integrating sphere. Transflection thus measures

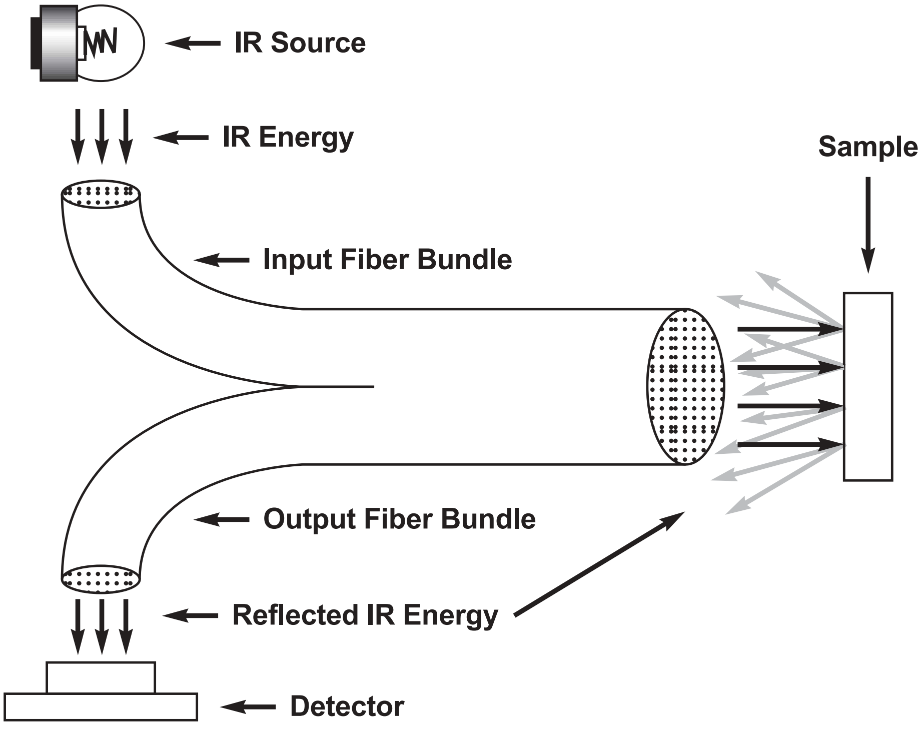


Figure 9. Schematic diagram of a diffuse reflectance probe.

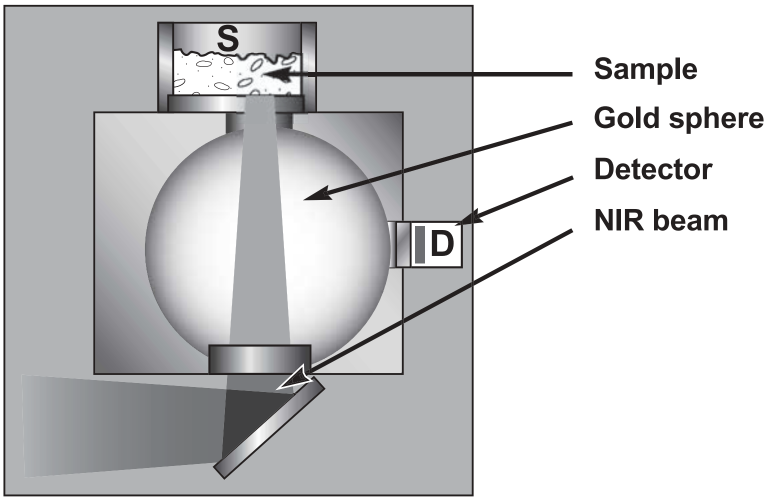

S
Sample
Gold sphere
Detector
NIR beam
D

Figure 10. Schematic representation of an integrating sphere.

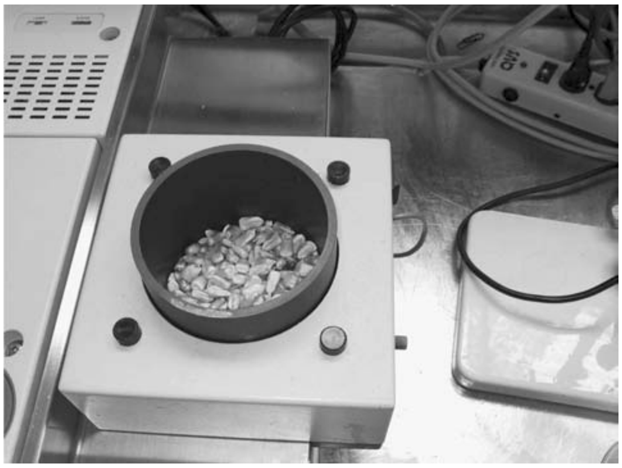


Figure 11. A rotating cup full of whole kernel corn on an integrating sphere.

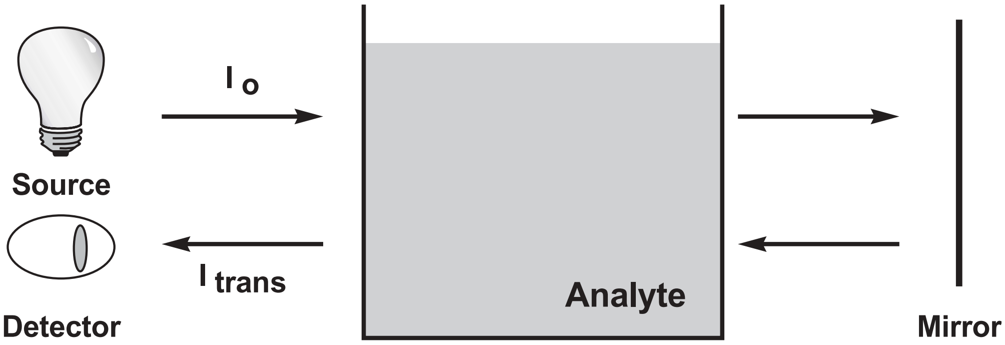

I_o
Source
Detector
I_{trans}
Analyte
Mirror

Figure 12. Schematic diagram of transflection.

a combination of transmission and reflection. This technique is useful for emulsions, gels, and liquids.

Calibration development

It is very important to acquire the proper instrument and sampling accessories as it directly affects the next stage involving NIR technology, which is calibration development. When using a NIR spectrometer, a large amount of time is initially spent turning the raw spectra into useful information. The ultimate goal is to be able to predict component concentrations in a matter of seconds or minutes, so the time spent performing wet chemical analysis of the calibration samples is well worth the effort.

THEORETICAL BASICS

When infrared radiation passes through a sample, some of the light is transmitted or reflected, without interacting with the molecules, and the remainder interacts with the molecules and is absorbed. The wavelengths at which the absorption will occur and how much light will be absorbed depend on a given substance. This is the basis of qualitative and quantitative analysis by spectroscopy, including NIR analysis. The amount of absorbed light is measured in absorbance. The absorbance, generally denoted as A, is defined as:

$$A = -\log (I_s/I_0)$$

Where I_0 is the light intensity before passing through a sample

I_s is the light intensity after passing though the sample

Spectroscopic quantitative analysis is based on the Beer-Lambert law (also know as Beer's law), which states that there is a linear relationship between the absorbance and concentration of absorbing molecules. Mathematically, Beer's law can be written as:

$$A = \varepsilon C D$$

where A = Absorbance at a specific wavelength
ε = absorption coefficient at a given wavelength
C = concentration of absorbing molecules
D = pathlength

Since the pathlength D is often fixed, in practice the absorption coefficient and the pathlength are combined into one coefficient k. Equation (1) can be then simplified to:

$$A= kC \qquad \text{(Equation 1)}$$

Solid or slurry samples are often measured in diffuse reflectance mode, using the accessories shown in Figures 9 and 10. For reflectance measurements log (1/R) is used instead of absorbance, where R denotes reflectance. This is defined as:

$$\log (1/R) = -\log (I_s/I_0)$$

Where I_0 is the light intensity measured from a reference material

I_s is the light intensity measured from the sample

The reference material used in the NIR region is usually a material with uniform scattering properties and very little absorption over the entire NIR region. Two kinds of material are used commercially. One is called Spectralon®, a material made of a special type of Teflon®; the other one is diffuse gold, a piece of metal with finely roughened surface coated with gold.

There are many theories regarding quantitative analysis in diffuse reflectance measurements. Empirically, log (1/R) is found to be fairly linear with the concentrations of chemical components in the NIR, hence,

$$\log (1/R)= kC \qquad \text{(Equation 2)}$$

The similarities between equations 1 and 2 are apparent.

Quantitative analysis generally has two steps: calibration and prediction. During the calibration stage, NIR spectra of a set of samples will first be measured so that the absorbance values in

Equation (1) can be determined. The concentrations of the chemical components can be analyzed with traditional lab methods such as wet chemical or HPLC methods. Once the absorbance values A and concentrations C are known, the values of the coefficients k can be determined by the least squares method. Once the coefficients are determined, the spectrometer is calibrated and ready to predict the concentrations of unknown samples. Equation 1 only works in a simple system where the absorption at a particular wavelength comes from only one component. Such a method is often called univariate calibration.

As described earlier, NIR spectra are highly overlapped, meaning that absorbance at a particular wavelength contains contributions from multiple chemical components in the sample. The univariate calibration can therefore rarely be applied to NIR applications. A more sophisticated multivariate analysis is usually required to build the calibration model. When multiple components are present, the total absorbance at a given wavelength is the summation of absorbances of each individual component. The absorbance at a given wavelength can then be expressed as follows:

$$A = \varepsilon_1 C_1 D + \varepsilon_2 C_2 D + \ldots + \varepsilon_n C_n D \quad (2)$$

where

A = Total absorbance at a specific wavelength
ε_1 = Absorption coefficient of component 1
C_1 = Concentration of absorbing component 1
D = path length
ε_2 = Absorption coefficient of component 2
C_2 = Concentration of absorbing component 2
ε_n = Absorption coefficient of the n^{th} component
C_n = Concentration of absorbing component n

There are four multivariate methods used to build the calibration model, Classical Least Squares (CLS), Multiple Linear Regression (MLR), Principle Component Regression (PCR) and Partial Least Squares (PLS). How these methods work is beyond the scope of this chapter, but there are excellent review articles describing how all these methods work as well as the pros and cons of each (Haaland, 1988; Martens *et al.*, 1989). PLS is the most commonly used method for building multivariate quantitative models and is the method used in all data analysis presented in this chapter. This chapter will only deal with practical aspects of development of calibration models and how to interpret common terminology used in the PLS method.

CALIBRATION SAMPLE SET, NIR MEASUREMENTS AND REFERENCE ANALYSIS

To build a quantitative calibration model using NIR data, the user must have a reference analysis method to determine the concentrations of the components of interest in a sample. The reference methods commonly used in QC laboratories in the distillery industry today are HPLC, GC, DMA and various wet chemical analyses. The accuracy and precision of a NIR calibration model is strongly affected by the quality of the reference analysis method. A few examples are given in the section of this chapter dealing with NIR applications in the distillery. The reference methods should be closely examined and understood so that the user does not have unrealistic expectations of NIR calibration models.

When building the calibration model, the reference analysis and NIR measurements should be performed as close to the same time as possible if samples are not stable. For example, live fermented mash samples taken from a fermentor are unstable. The longer a sample sits between the time of NIR measurement and the time of the reference measurement, the bigger the error to be expected.

Preferably, samples from production that span a period of time should be incorporated in the calibration model, with the goal of incorporating all possible variations in the production process. One must be cognizant of production parameters, and collect unique samples when they occur. For example, DDGS can vary in a whisky distillery with the many different mash bills, which can include grains such as rye, barley, wheat, and corn. The NIR user must recognize when different mash bills are being fermented and collect samples that do not occur very often and incorporate them into a calibration model. This means it may take an extended period of time to obtain a representative set of samples, but it is important to do this because it makes a calibration model more robust, accurate, and precise. Another occasion when a user should incorporate unique samples is when processing equipment breaks down. Usually these are times when unique or extreme samples occur. For example, when a belt conveyer breaks while delivering corn to a pre-mix tank it can cause mash to be more dilute than usual. The user can collect the mash in these fermentors for analysis, collect the syrup in the evaporators, and collect the DDGS at the end, because the broken conveyer affects all of these processes. In that way, the next time the conveyer malfunctions the NIR user can quickly identify the source of the problem. To make the calibration model more robust, it is recommended that any odd samples be included at least three times in the calibration model (Williams and Norris, 2001).

The calibration samples should also span the concentration range as smoothly as possible, avoiding large numbers of calibration samples at just a few concentrations. For example, refer to the calibration model for crude protein in DDGS in Figures 13a and Figure 13b. In Figure 13a there are not enough samples of DDGS with low (21 to 22%) and high crude protein (27 to 31%). To make a calibration model more robust and precise, the sample distribution should be similar to that of Figure 13b. This type of sample distribution, where samples are evenly distributed over the calibration set, is often described as a 'boxcar' effect (Williams, 2001).

The calibration range should be larger than the specified range and not too small in view of the error of the reference method (EMEA 2003)**.** If the required accuracy is greater than 0.1% by weight, generally it can be achieved with NIR. If the required accuracy is between 0.1 to 0.01% by weight, then the NIR application is likely to be feasible for a simple system with very few components, an accurate reference method, a wide enough concentration range, and a lot of samples. If the required accuracy is 0.01% wt to a few ppm, the application is unlikely to be feasible.

BUILDING CALIBRATION MODELS

After the calibration samples are collected, measured by a NIR spectrometer and analyzed by the reference method, the user can then develop and validate the quantitative calibration models. Once the calibration model is validated, the user can finally enjoy the fruits of all this labor: the prediction of multiple parameters from a single NIR measurement in a matter of seconds to minutes. Validation is the key to the development of PLS models. Validation is used both to optimize many parameters during the development of the model, and to identify abnormal samples (outliers). There are two types of validation: cross validation and external validation or test-set validation.

External validation

External validation is sometimes referred to as test-set validation. The sample set used in external validation consists of samples with known reference data that were not used in the calibration set for the model development. The goal for the external validation is to test the predictive ability of the model. At the end of external validation, the NIR predicted values are compared with those from the reference method, often expressed in a plot of NIR predicted vs. reference values. The value of R^2 in this plot is one of two important indicators for how good the model is. The other one is the Root Mean Square Error of Prediction (RMSEP), which is used as an indicator of the average error for NIR prediction on future unknown samples.

Cross validation

The advantage of external validation is that it is a true independent validation. The main disadvantage of external validation is that it wastes the valuable calibration data in order to check the predictive ability of the calibration models. When the number of available calibration samples is small, a procedure called cross validation is often used. In a cross validation, the first calibration sample is removed from the calibration set. PLS models are generated using the remaining calibration samples. The PLS models are then used to predict the first sample that is left out and the error of prediction for this sample is calculated. The first sample is then re-included into the calibration set and the next sample is removed from calibration. The whole process is repeated

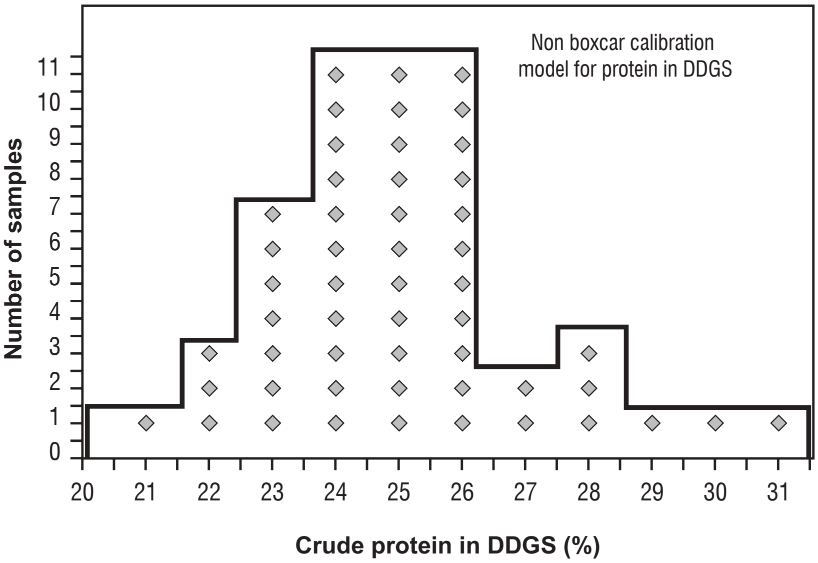


Figure 13a. A calibration set for protein prediction in DDGS.

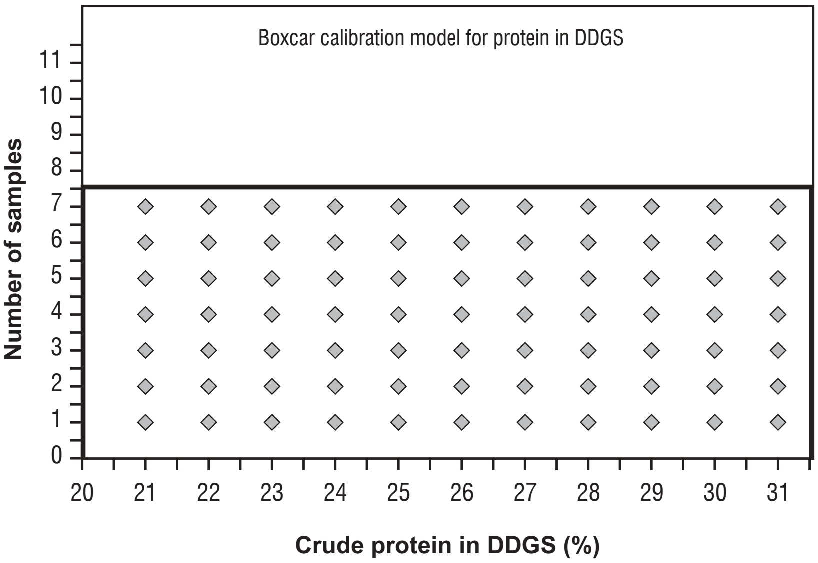


Figure 13b. A calibration sample set for protein prediction in DDGS.

until the last sample is removed and analyzed. The number of samples left out can be just one sample or a small set of samples. At the end of cross validation, the Root Mean Squared Error of Cross Validation (RMSECV) is calculated and used as an indicator of the future predictive ability of the models.

Results of validation

As discussed earlier, the cross validation or external validation result is often shown in a plot of NIR predicted values *vs.* reference method values. In an ideal situation, this plot should be a 45° line, where the prediction should equal the reference value. The closer the R^2 value is to 1 or 100%, the more accurate the model. Figures 14a and 14b show a PLS cross validation result and an external validation for prediction of ethanol concentrations in corn mash samples.

Errors exist in all measurements and cannot be avoided. It is important to identify the

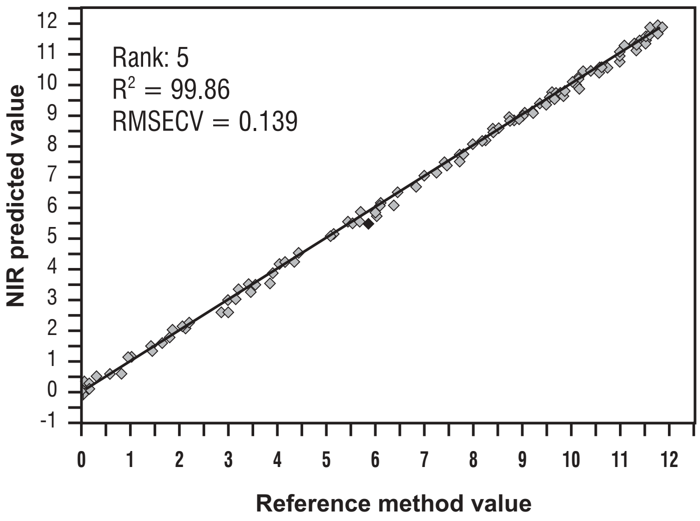


Figure 14a. Cross validation result for ethanol prediction in fermented corn mash samples. The values of R^2 and RMSECV were 0.9986 and 0.139 % v/v, respectively. The number of samples in the calibration set is 111.

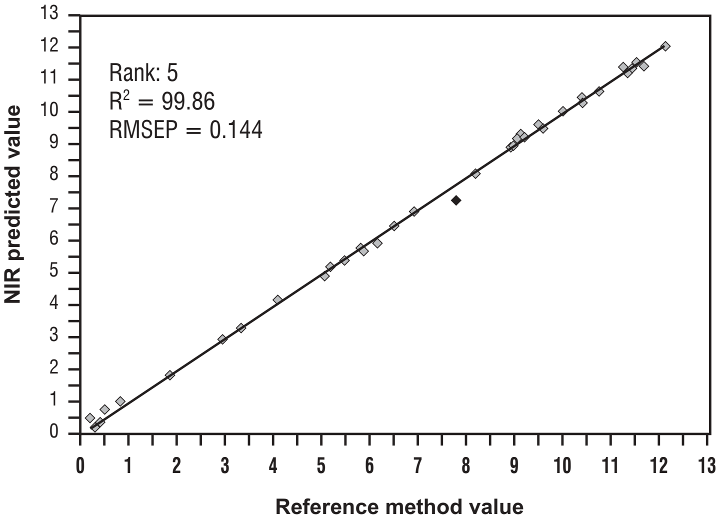


Figure 14b. External validation result for ethanol prediction in fermented corn mash samples. The values of R^2 and RMSECV were 0.9986 and 0.144 % v/v, respectively. The number of samples in the validation set is 40.

accuracy that is needed for a plant to keep a process under control. For example in protein in DDGS, there is no need to strive for an error of 0.05% when an error of 0.5% is satisfactory for plant operations; however, a 0.05% error for determining alcohol strength in a finished blend can cost a company money in terms of ethanol losses and taxes.

How many samples in a calibration data set?

It is very important to include an adequate number of samples in the calibration set. In general, one should incorporate as many as are available. Whether or not there are enough samples to build a stable calibration model can only be determined by performing validation tests with an independent validation set. As a rule of thumb, one should start with a minimum of 10 samples for each independent chemical component or other source of variation. ASTM provides guidelines that can be used to judge whether or not a calibration set contains sufficient samples (ASTM, 1997).

Calibration model updating

When a calibration model is finished, it is good practice to check periodically to ensure the validity of the answers (Martens *et al.*, 1989). The checks might be frequent when a calibration model is first used, but they will become more infrequent as the model becomes more robust over time. These routine checks are not only important in the maintenance of the calibration models, but also help build confidence in operators, technicians, and management, for whom NIR might be relatively new technology.

Distillery applications for NIR analysis

Distillery processes are ideal situations for NIR analysis since most compounds in a distillery are organic and at sufficiently high concentration. From the beginning, when grain is unloaded from the truck, to the end where product goes into a bottle, NIR analysis can provide useful information to maintain process control.

INCOMING GRAIN

Unlike fuel ethanol production where corn is 100% of the mash bill, in a Canadian whisky distillery corn is approximately 90% of the mash bill. It is the largest expense to a distillery, yet it goes largely unmonitored throughout the distilling industry. The components of importance to distillers are moisture, protein, oil, and starch, all of which can be measured by NIR spectroscopy.

Moisture content of incoming grain is of critical importance. If moisture becomes too high it can cause mechanical problems with a hammer mill and cause downtime in the distillery. Typically, a maximum specification of 15% moisture should be targeted. If moisture levels are above 15%, there is also less starch available to make alcohol. It is up to the distiller to either make concessions with the corn broker or to reject the load of grain. This type of monitoring can justify the expense of a NIR spectrometer.

Protein content of whole kernel corn is another parameter that can be analyzed, because it affects composition of DDGS, a valuable by-product at the end of distillation. For example, in 1998 a distillery was having problems meeting the guarantee for protein content in DDGS. At first, it was thought there were issues with fermentations not finishing properly; however on investigation it was found that fermentors were finishing with a good yield. After further investigation of the NIR crude protein data, it was found that the average protein level in the incoming corn fell from 7.5–8.0% in 1997 to 7.0% in 1998 due to drought conditions. It was found that the expected crude protein content in DDGS could be roughly determined by multiplying the protein content of incoming corn by 3. A difference of 0.5–1.0% in protein content of incoming grain will therefore affect the protein content of DDGS by 1.5–3%. There is nothing a distillery can do to change the incoming crude protein levels of poor quality corn. The only option is to lower the crude protein guarantee of DDGS.

Oil content can also be monitored by NIR spectroscopy. The reason that it may be important to measure oil is that genetic varieties of corn with high oil contents exist in the commercial market. Measuring incoming loads for oil allows the distiller to prevent high oil corn varieties from entering the system.

Starch is the component with the greatest effect on overall yield because it is the source of fermentable sugar for yeast growth and ultimately alcohol production. NIR spectroscopy can allow a distiller to track the starch levels in purchased corn.

There are several advantages to using NIR spectroscopy as a primary tool for determining the quality of corn:

1. The first, and most obvious is the rapid analysis of essential components. It can take up to three days to determine the levels of starch, protein, moisture and oil in one corn sample by traditional analysis methods.
2. The distiller can track corn suppliers more closely to prevent becoming the victim of fraud. The way samples are collected is important, because it can affect measurement results. The suggested method is to gather a composite sample from a truck with a probe that can measure the bottom, middle, and top of the load. Also, it is important to measure the front, middle, and back end of the truck.

A sample should always be collected by this consistent and statistically reliable method. Sampling with this method can also detect loads containing grain that is out of specification on the bottom that have been 'topped up' with good quality grain.

3. Production efficiencies can be tracked more closely. Accurate determination of production efficiency is important, because it helps the distiller make improvements and reduce costs.
4. More crop data accumulated each year.
5. Variety selection for desired characteristics is possible.

The calibration model Hiram Walker & Sons currently uses to monitor incoming corn was provided by Bruker Optics with the purchase of the NIR spectrometer. The expected value of each of the four major components and the predictive error of each component expressed in RMSEP are listed in Table 1.

Table 1. Expected values and predicted errors for starch, oil, protein and oil content of incoming corn expressed in RMSEP.

Component	*Expected result (% as is basis)*	*Approximate RMSEP (+/- %)*
Starch	60–62	1.2
Oil	2–3	0.5
Protein	7-8	0.5
Moisture	12-15	0.3

A major advantage of using an FT-NIR system is that it has high wavelength accuracy and precision, which allows calibration models to be easily transferred from instrument to instrument via the internet, without having to adjust for slope or bias on each instrument. As a precaution, the downloaded models are validated with a set of 40 corn samples that are not from the original calibration set. The predicted error of the 40 validation samples measured by the instrument in the Hiram Walker & Sons plant was roughly the same as the error in the original model, which met requirements.

Another advantage of this calibration model is that reliable results can be obtained by measuring incoming corn directly without any sample preparation such as grinding. Since the absorption coefficient in the NIR region is low, NIR light can penetrate corn kernels and whole kernels can therefore be used for model development. The NIR spectrum can be collected with the aid of an appropriate sampling device such as a rotating cup or a flow-through funnel. Depending on the available sampling devices and how a particular calibration model was developed, some instruments today still require users to grind incoming grain in order to achieve a reliable measurement.

Calibration models for other grains including rye, rye malt, barley, and barley malt are currently being developed. The components of interest include starch, oil, protein, moisture, and enzymatic activity.

FERMENTATION MONITORING

Fermentation optimization can be complex, because many parameters can affect the final alcohol content and overall yield. With the traditional analyses available today it can be time consuming for laboratory staff to keep up with the demand for fermentation analysis. Optimum efficiency can be achieved if all parameters are kept in control prior to and at fermentation, but this does not always happen as equipment failures can occur hours before being identified and corrected. NIR technology can identify non-optimum conditions in the fermentation almost immediately, allowing correction of problems. In addition, NIR analysis can aid distillers in evaluating the new enzyme and yeast nutritional supplements.

In the industry today, most distilleries use an HPLC for fermentation monitoring, including measurements of sugars, acids, glycerol, and alcohol. All these components are interrelated and it is very important to keep track of them at each stage of fermentation, but there are disadvantages to using HPLC as the primary monitoring tool:

1. The technician must be highly skilled.
2. Accessories can be quite expensive (i.e. columns, chemicals, filters).
3. The amount of time needed to determine the

correct answers is at least 20 minutes. This does not include repeat samples, sample preparation time, technician training time, instrument setup time, wash outs, and calibration time. This can be frustrating to fermentor operators when quick decisions are required.

4. The sample preparation for HPLC only permits analysis of those components that are soluble in the liquid fraction. To extract the components of interest from the mash, it must be centrifuged or filtered to retain the liquid portion. This will compound the error because the solid portion that is removed has sugars, acids, and alcohols still bound to the solid particles, thus skewing results.
5. Results are not always as accurate as required.

The amount of ethanol produced in fermentation is very important, and the potential improvements in fermentation efficiency that NIR technology can support should not be underestimated. For example, if a fermentor is 200,000 liters and the average fermentation finishes when alcohol content reaches 9.6%, this means that the plant will produce 19,200 liters of absolute alcohol per fermentor. After researching different protocols to optimize fermentations such as changing enzymes, process parameters, or nutritional supplements, the alcohol content was raised to an average of 10.6% per fermentor. This means that 21,200 liters of absolute alcohol are now made in each fermentor. If 1000 fermentors are set per year in a plant to produce a certain amount of alcohol, then one percentage point increase in fermentor alcohol content means that plant can now set only 905 instead of 1000 fermentors to produce the same volume of alcohol. This means considerable savings in raw materials, processing fuel, steam, labor, maintenance, and equipment. Also, plants strive to be energy efficient, and generating steam can be quite expensive. Overall, this can mean millions of dollars saved annually for a distillery. Figure 15 shows the results of a number of fermentation optimizations monitored by NIR spectroscopy at Hiram Walker & Sons distillery over a four-year period. NIR has proven invaluable in assisting in fermentor optimization and in saving energy costs.

It is clear from the example above that raising the percent alcohol in a fermentor by even 0.1% can increase efficiency and contribute to cost savings for a distillery. There are two reference methods that can be used to calibrate the NIR model, HPLC or distillation - DMA. The NIR model should be based on the most accurate

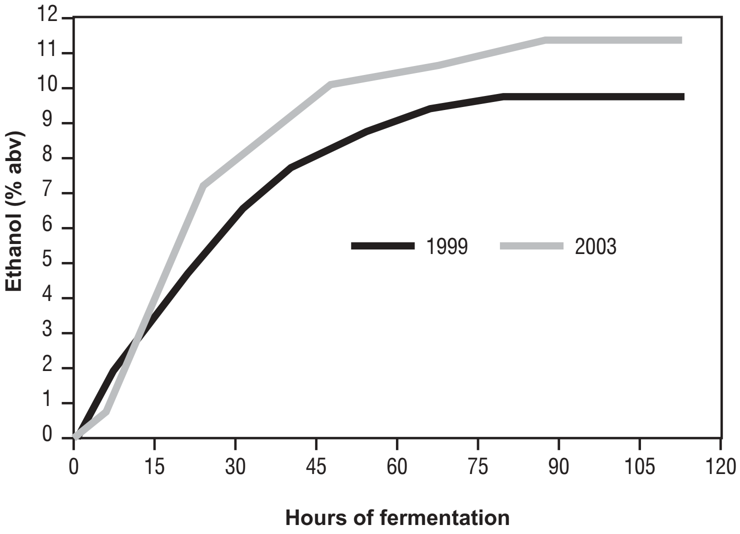


Figure 15. Fermentation profiles from 1999 to 2003 during which period NIR has assisted in optimizing fermentors and thus improved the distillery bottom line.

reference data available in order to determine when an enzyme, nutritional supplement or other change in fermentation conditions is effective.

Figures 16a and b show the two calibration models for determining ethanol in corn mash. The first NIR model was built using HPLC as the reference method (Figure 16a) and the second was built using the distillation – DMA reference method (Figure 16b). The distillation – DMA method is to distill 100 mL of the corn mash, collect 100 mL of the distillate and then determine the percent alcohol via the DMA. The value of RMSECV for the NIR models using the HPLC and distillation-DMA method is +/- 0.67% and 0.18%, respectively. This demonstrates the greater accuracy of the distillation-DMA method for the calibration of NIR models for corn mash. It is recommended that the distillation-DMA procedure be used for ethanol calibrations in corn mash instead of the HPLC method.

In contrast to alcohol, for sugar calibration models HPLC is recommended as the reference method. Even though the prediction error for sugar is roughly +/- 0.5%, it is still acceptable. The only concern to a distiller is that sugar is

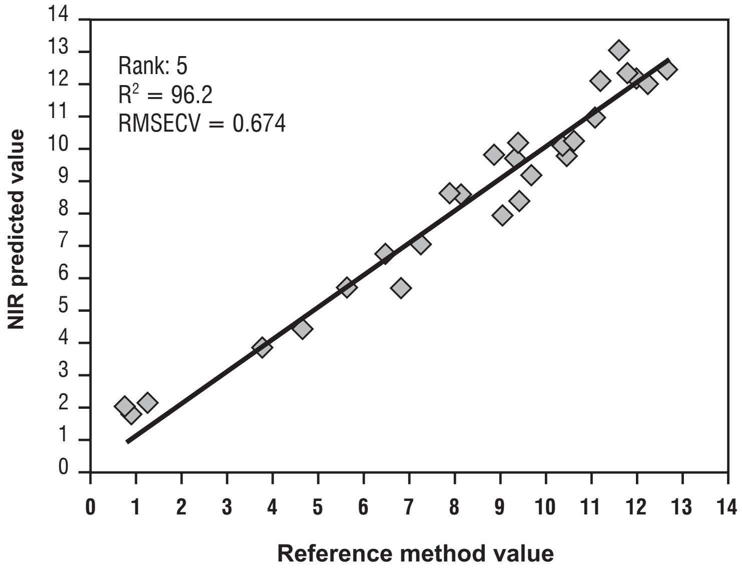


Figure 16a. Cross validation result for ethanol content in 30 fermented corn mash samples. The reference method of analysis was HPLC. The values of R^2 and RMSECV were 0.962 and 0.67% v/v, respectively.

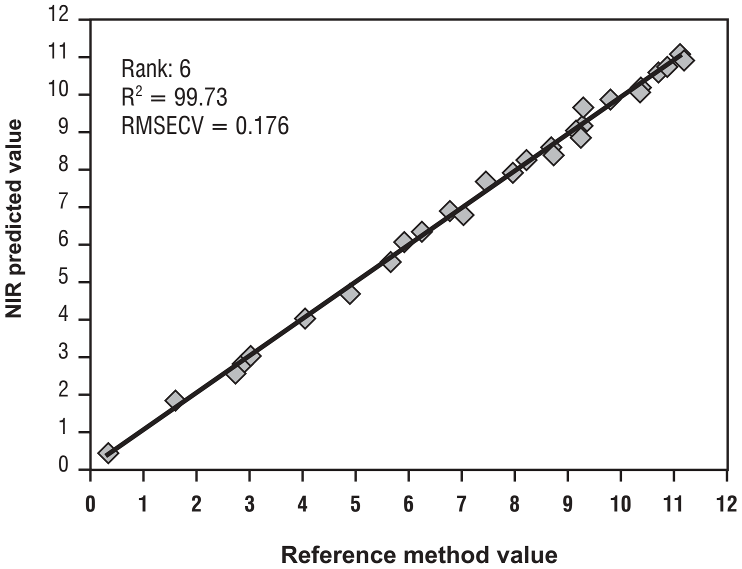


Figure 16b. Represents the cross validation result for ethanol content in 30 fermented corn mash samples. The reference method of analysis was by distillation - DMA. The values of R^2 and RMSECV were 0.997 and 0.18% v/v, respectively.

being converted and is used up by the yeast. If sugar values are not decreasing then the fermentation parameters must be quickly investigated and proper actions taken to correct the problem. Figure 17 shows the carbohydrate concentration over a 140 hr period, monitored using NIR spectroscopy.

Another important parameter in corn fermentation that NIR spectroscopy can monitor is the production of lactic acid. The primary method of calibrating the NIR for lactic acid is HPLC. Lactic acid is a compound produced by *Lactobacillus* sp., bacteria which compete with yeast for sugar. The more lactic acid produced, the more bacterial contamination is present. Figure 18 illustrates the amount of lactic acid produced over time in a typical batch fermentation process. It was noted that as the backset stillage use rate increased in the fermentor, so did the starting level of lactic acid. By monitoring lactic acid, a distiller can therefore adjust backset stillage rates in order to optimize fermentations. For continuous fermentations, the lactic acid values can be used to determine when antibacterial products are required or wash out procedures should commence.

A potential application for NIR technology is optimization of fermentor cleaning. One can optimize the amount of wash water and detergent necessary for clean fermentations by correlating the wash out procedure with the levels of lactic acid produced at the end of fermentation, thus determining which wash out procedure is most cost effective.

NIR spectroscopy is a flexible analytical tool because its results are based on the best primary methods available, distillation – DMA for ethanol, and HPLC for sugars and lactic acid. The predictive errors of the calibration models currently used to monitor corn fermentations at the Hiram Walker & Sons plant are shown in Table 2.

Table 2. RMESCV values for fermentation parameters.

Component	*Approximate RMESCV*
Ethanol	0.14
Dextrins	0.50
Dextrose	0.46
Maltose	0.52
Lactic acid	0.11
Glycerol	0.07

Troubleshooting is one of the largest paybacks for an NIR spectrometer. Over the course of one

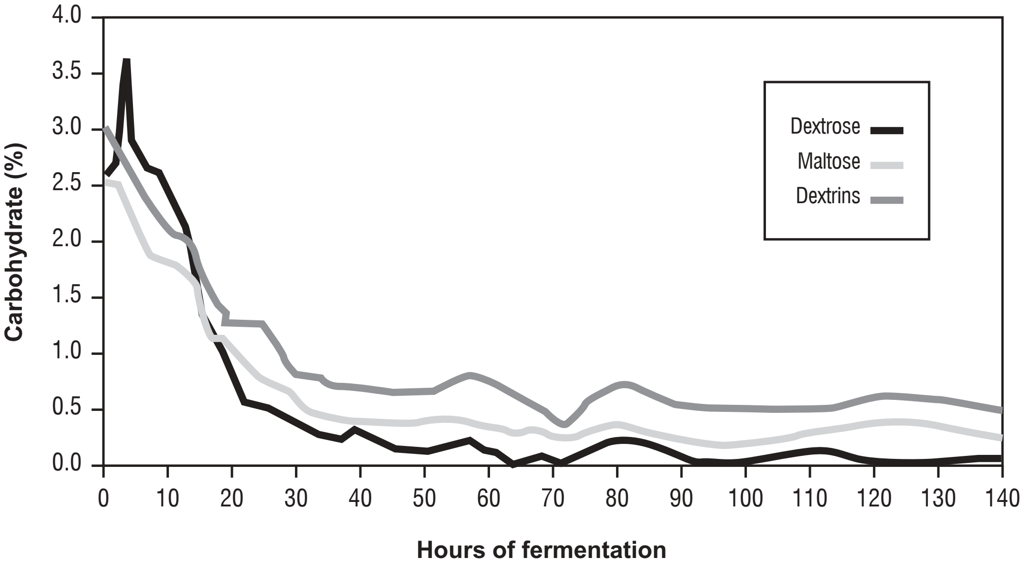


Figure 17. Typical reduction in carbohydrates in corn fermentations over a 140 hr period.

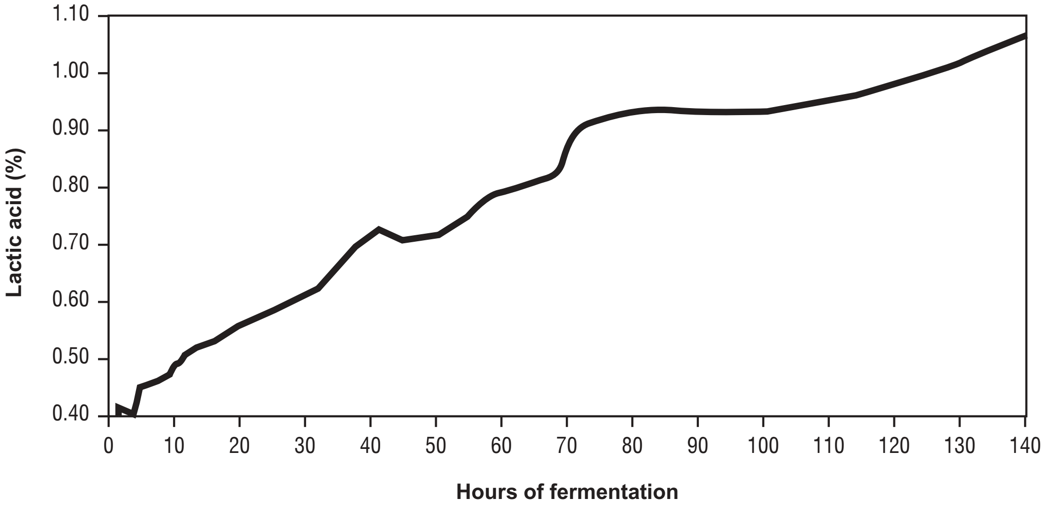


Figure 18. Production of lactic acid during the course of corn fermentations.

year, one lab technician can measure 5600 corn mash samples with just one NIR spectrometer. There are 30 batch fermentors at the Hiram Walker & Sons facility, and all are kept full of mash until the mash is sent to the beer well. Today Hiram Walker & Sons laboratory monitors the fermentors at 12–40 hr intervals and can predict the final percent alcohol. If ethanol values are lower than expected, a course of action can be determined. Troubleshooting of fermentors involves checking records for correct enzyme or yeast addition, or auditing the plant for mechanical failures. An example of mechanical failure is an increase in lactic acid values and a decrease in ethanol in one fermentor, in which case the Butterworth washer must be closely inspected to determine if it is rotating properly. As another example, if the alcohol content is too low, check for small leaks in the cooling coils. NIR technology can assist in identifying and fixing problems in the early stage before yield is compromised.

The use of NIR spectroscopy for fermentation analysis allows the distillery to rapidly perform research on fermentation supplements. One example is experimenting with various forms of nitrogen. Nitrogen is the limiting substrate for yeast growth in fermentations. Figure 19 shows NIR results from nitrogen supplement experiments. It was determined that nitrogen at a specific level decreased fermentation time (increased rate of fermentation) by 30 hrs. A fermentor with a nitrogen supplement added finished in 50 hrs while the control fermentors finished in 80 hrs. A downside of the nitrogen supplement, however, was that if the control fermentors were given enough time, they would finish with more alcohol than the nitrogen-fed fermentors. This is because the nitrogen induced yeast cell growth from the carbohydrate source instead of alcohol production. In the end, it was not economical for this distillery to use nitrogen as a supplement, since 80 hrs of fermentation was acceptable. However, if production capacity ever needed to be increased, or if the gravity of fermentation was increased, then a nitrogen supplement could be an asset to this operation. The NIR analysis allowed the distillery get analytical results quickly, utilize personnel to their maximum potential, and optimized production.

NIR is an asset in determining the efficiency of fermentations. For example, incoming corn was monitored by NIR spectroscopy and found to contain 62.6% starch (as is). This means that there were 626 kg of starch per metric tonne of corn. The 626 kg of starch should produce 696 kg of dextrose if it were completely converted. The theoretical ethanol yield from yeast is 51.1% ethanol; therefore, the theoretical yield from 1000 kg of this grain is 451 liters. If scales are in place to measure the amount of corn that enters the fermentor, then the efficiency of fermentations can be calculated. If a 200,000

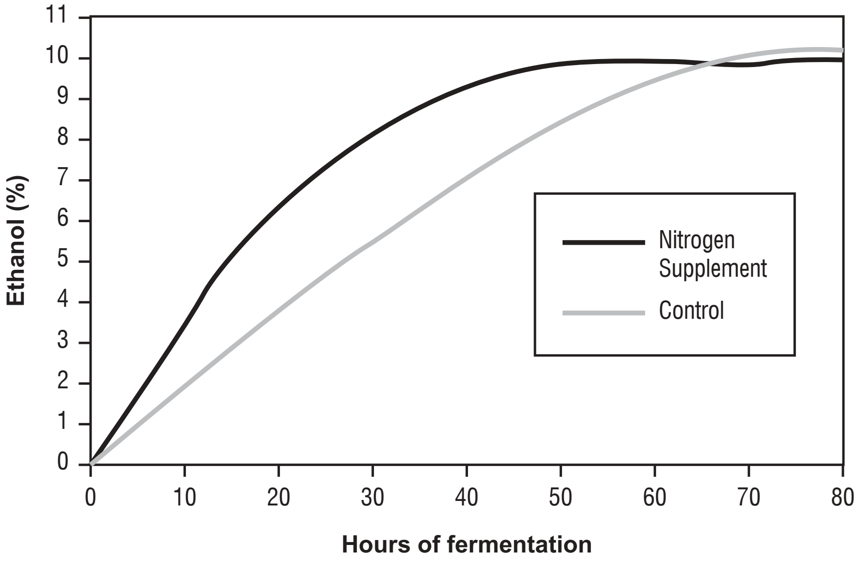


Figure 19. Effect of a nitrogen supplement to corn fermentations on fermentation time.

liter fermentor contains 50 metric tonnes of corn and finishes at 10.0% alcohol, then final yield is 400 liters of absolute alcohol per tonne of grain. But theoretical yield is 451; therefore, this fermentation was 89% efficient.

The efficiency of the distillation column can also be determined. If the above fermentor contained 20,000 liters of ethanol, and the tank that recovered the ethanol contained 20,500 liters at 96.0% ethanol, then the distillation efficiency was 98.4%.

Calibration models for rye mash, barley mash, and molasses have also been developed at Hiram Walker & Sons distillery. Optimization studies, efficiency determinations, and troubleshooting currently are carried out for these types of fermentations. A future calibration model also possible using NIR technology is determination of the percent solids in mash prior to exiting the pre-liquefaction tank or prior to fermentation. This can help quickly identify if there are milling problems or belt scale problems.

DRYHOUSE OPERATIONS

There are several applications for NIR analysis in the dryhouse. The first application is the determination of the percent solids in solubles, or the condensed syrup discharged from the evaporators. After the third effect of a falling film evaporator, the desired level of solids is around 40%. If the solids level gets much higher, it can cause fouling and eventual plugging of the discharge lines. It is very expensive to clean pipes plugged with solid material; therefore it is highly desirable to have a quick and easy method to determine the percent solids. NIR has the ability to measure the percent moisture of the syrup in a Pyrex beaker at very warm temperatures with the integrating sphere accessory. After the percent moisture is determined, simply subtract this value from 100 to get the percent solids. At Hiram Walker & Sons, the RMSECV for this calibration model is +/- 0.8%.

The most important application for NIR analysis in the dryhouse area is DDGS analysis. After the rotary driers, most distilleries will guarantee to their customers minimum levels for protein and fat; while there are maximum levels for moisture, fiber, and ash. Protein levels for DDGS are probably the most important guarantee for the end customer. The most accurate wet chemical method for protein is the Kjeldahl method. Figure 20 illustrates the cross validation for a calibration model using the Kjeldahl method as the reference method.

Moisture analysis is also important for the quality of DDGS. To get optimum results, the most accurate wet chemical procedure for moisture analysis is the Bidwell-Sterling method, as compared to the oven moisture method. The procedure for the Bidwell-Sterling method involves refluxing 50 g of DDGS in 200–250 mL of xylene for 2 hrs. There is a graduated side arm that collects the water (more dense) from the DDGS, and the remaining (less dense) xylene falls back into the boiling flask. At Hiram Walker & Sons the RMSECV of the NIR calibration model for moisture using Bidwell-Sterling as the reference method is +/-0.35%.

The three remaining components in DDGS that can be measured by NIR are fat, fiber, and ash. A fat extraction unit is used to obtain the reference value for fat content in DDGS, which is a 2-day test. Similarly, fiber content is determined with a fiber extraction unit, which requires three days. These two components will take an NIR technician a much longer time to develop the calibration models. In addition, the cost of each of these extraction units can be quite high and they require hazardous chemicals. The reference method for ash is a simple test. A 2 g sample is burnt in an oven for 2 hrs; and the weight loss is measured at the end. The RMSECV obtained at Hiram Walker & Sons for each DDGS component is listed in Table 3.

Table 3. RMSCV for DDGS components.

Component	*Number of factors*	*Approximate RMSCV (+/- on a % as is basis)*
Protein	7	0.46
Moisture	5	0.35
Fat	6	0.35
Fibre	5	0.49
Ash	6	0.13

A future opportunity for NIR analysis in the dryhouse is the determination of lactic acid in the backset stillage that is recycled to the fermentors. The toxic effects of backset stillage on yeast are well documented. If a distiller can monitor lactic acid and control the amount of backset stillage in the mash bill, then alcohol production can be optimized.

Another opportunity for NIR analysis in the dryhouse is to measure the residual starch in DDGS. After some initial preliminary trials with starch analysis by cooking, converting, and measuring sugar levels, it was found that this method can be quite complex, lengthy, and inaccurate. If fermentations are good, then residual starch will not be a concern. Time is better spent optimizing fermentation than creating calibration models for starch in DDGS.

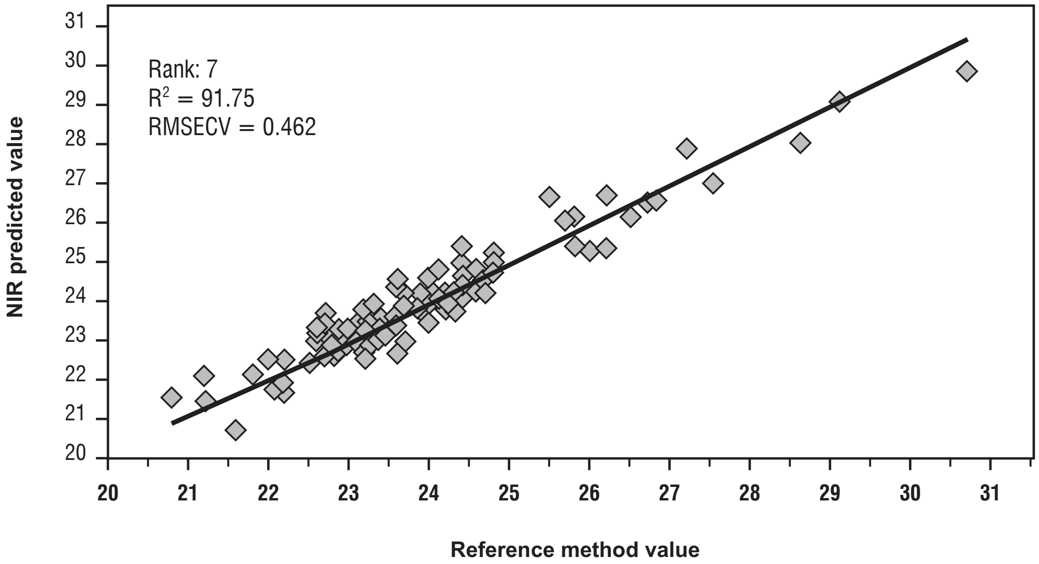


Figure 20. Cross validation of crude protein content of DDGS.

BLENDING OPPORTUNITIES AND ETHANOL DETERMINATION

In the whisky or liqueur industry it is imperative that the finished product contain the correct strength of alcohol. Most products will have a target specification. For example, most whiskies have a specification of 40.0% alcohol by volume, however, governments have tolerances on these specifications. For the Bureau of Alcohol, Tobacco and Firearms (BATF), the specification is 40.0% minus 0.15% alcohol by volume, which gives a very small target range of 39.85-40.00%. Excise Canada has a larger tolerance window of 40.0% ±0.2%, which is a target range of 39.8%-40.2%. This leaves a very tiny window for a blender to make sure of the correct proof of the final product.

To make matters more difficult, when blending whiskies, rums, tequilas, or liqueurs there are suspended solids that obscure the final percent alcohol when measured by a DMA. The obscuration has to be determined prior to final proofing in order to determine the true percent alcohol. In order to compensate for the obscuration, the blender must either distill the sample and proof the distillate by DMA, or oven dry the alcohol and determine the solids by weight and factor it into the 'as is' DMA result. Either method can be time consuming, and can hold up production for hours for the true percent ethanol reading. If the true percent ethanol reading is outside the tolerance reading, then the blender must make adjustments and repeat the process. Laboratory analysis in a blending department is the bottleneck of the operation, and the longer the product stays in a tank the more money it costs the company. In order to reduce the time in blending, the NIR technology can be a considerable asset. Currently NIR measurements are collected using 8 mm disposable glass vials in transmission mode; however a fiber optic probe can be used if desired.

Another benefit of NIR analysis is that it was found that the spectrometer could be calibrated to measure ethanol over various temperature ranges without losing much accuracy. This is possible only if data taken at different temperatures are included in the model. Extra samples should be included in the calibration when failures in the environmental controls of the laboratory occur. The temperature effects on NIR spectra and the NIR calibration model for ethanol in a liqueur were observed during a period when the Hiram Walker & Sons laboratory temperature control system malfunctioned. The normal liqueur samples were often detected as abnormal samples (outliers) during this period. To resolve the issue, NIR spectra of the liqueur samples were measured over a range of temperatures and incorporated into the calibration model. This calibration model is shown in Figures 21a and b. Today, the calibration can be used to determine percent alcohol of the liqueur accurately even when the laboratory temperature changes a few degrees. Alcohol content of a liqueur can be calibrated to an RMSEP of +/- 0.036%, which is inside the tolerance range allowed by the BATF.

To illustrate further the importance of the reference method, the alcohol content of approximately 40 whisky samples was measured by three different methods: the DMA, the GC, and HPLC. These data were used to generate NIR calibration models. The results are shown in Figures 22a, b and c. This clearly shows that the DMA is the most accurate and reliable method for a blending department, and should be used as the reference method for NIR calibrations.

For the future, the next step is to implement in-line measurements to determine the proof of the spirit. Currently in a blending operation, the spirit is brought in house at high proof, and then tested for strength. Components are mixed and again tested for strength. The sample is then filtered for final polishing, then stored in a third tank. Water is the last component to be added to bring the product down to the final strength, which requires even larger tanks. If NIR were an approved method of determining alcohol strength, it could be used in-line as a method for alcohol strength in the dilution process immediately before bottling, thus requiring less tank space and reducing the associated costs.

An additional benefit of NIR technology is that different components can be determined from one measurement. For example, Figure 23 shows the measurement of titratable acidity for an alcoholic spirit with added malic and citric acids. The problem with this product was that when it was distilled for proofing, it required an additional prior step of neutralization in order

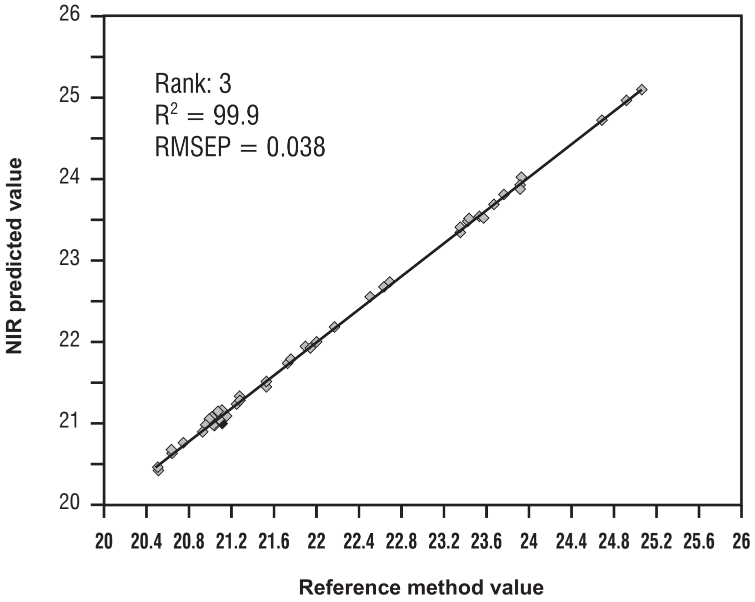


Figure 21a. External validation of the initial model for prediction of percent ethanol on an independent set of 94 liqueur samples. The values of R^2 and RMSEP for the external validation are 0.9990 and 0.038% vv, respectively. The number of calibration samples is 98.

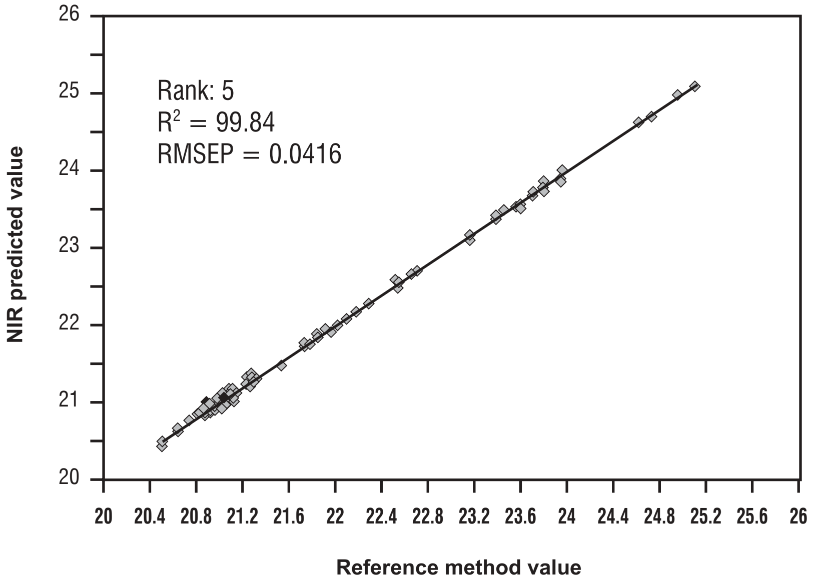


Figure 21b. The external validation result after incorporating additional variances such as temperature. The values of R^2 and RMSEP for the external validation are 0.9984 and 0.042% vv, respectively. The number of samples in the calibration set and independent validation sets are 123 and 120, respectively.

to achieve accurate results, which added significant time in the laboratory. NIR spectroscopy can measure the titratable acidity and the proof of the product at the same time. Other examples of components that could be analyzed with NIR spectroscopy are fructose or sucrose when blending liqueurs.

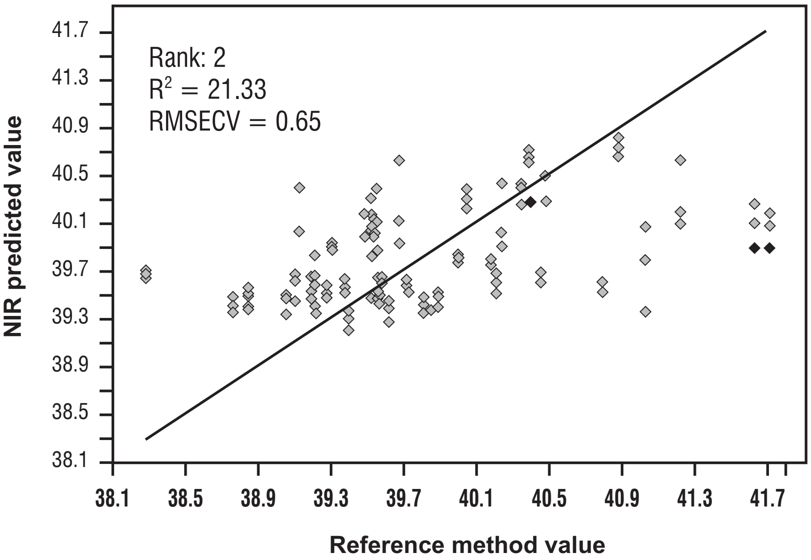


Figure 22a. Cross validation of 45 whisky samples where the reference percent alcohol was measured by HPLC. The RMESCV +/- was 0.66% v/v and R^2 is 0.213.

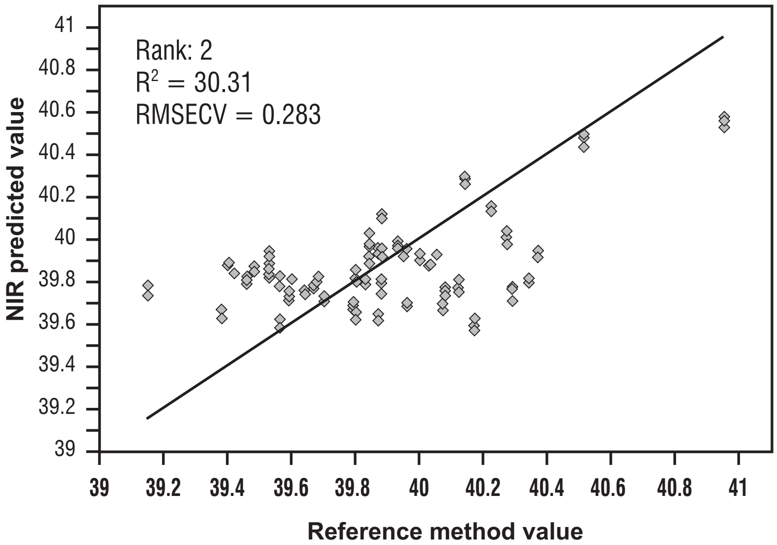


Figure 22b. Cross validation result of 45 whisky samples where the reference percent alcohol was measured by GC. The RMSECV was +/- 0.28 and the R^2 is 0.303.

Conclusions

NIR is definitely a powerful tool that should not be ignored in the distilling industry. Water and common organic molecules such as starch, protein, oil, fiber, ash, acids, sugars, and ethanol can be monitored quickly, easily and reliably by NIR spectroscopy at every stage of the operation. The quick and reliable NIR analytical result can help the distilling industry keep production processes in control. It is a tool of the future that will replace some of the older and slower techniques. NIR technology can also assist in research projects where more analyses can be done with fewer resources. NIR analysis allows distillers to troubleshoot and optimize

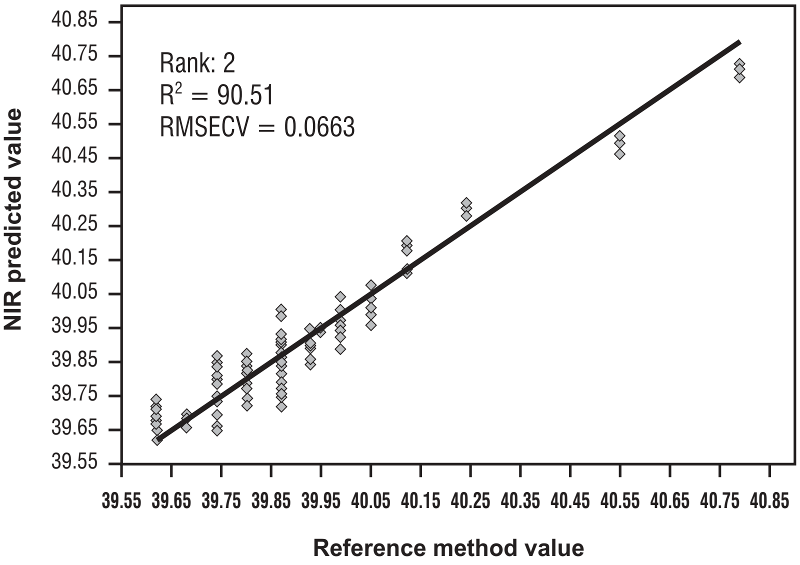


Figure 22c. Cross validation result of 45 whisky samples where the reference percent alcohol was measured by DMA. The RMSECV was +/- 0.067 and the R^2 is 0.905.

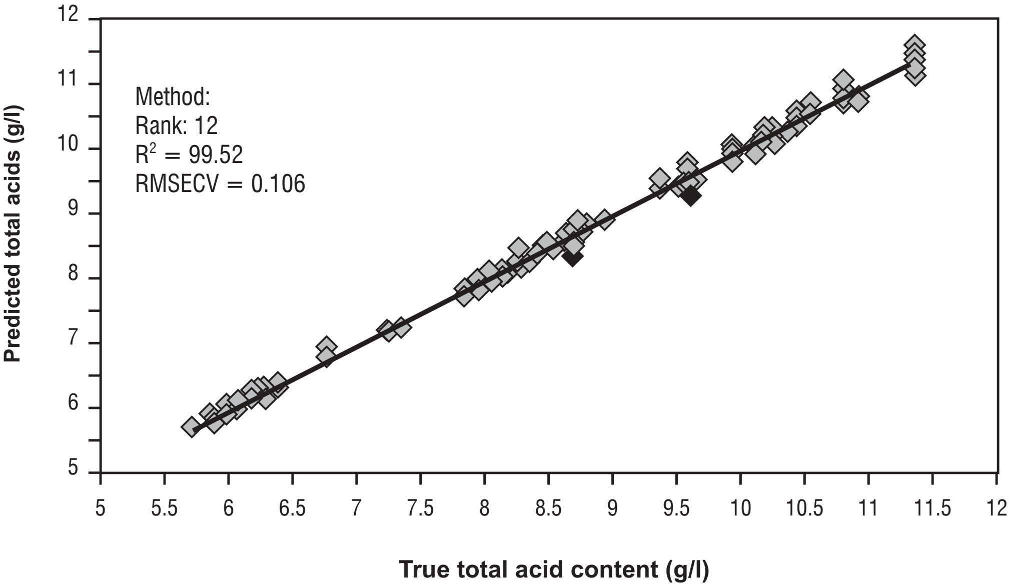


Figure 23. Cross validation of the titratable acidity of a high proof alcohol sample. The RMSECV is +/-0.106 with an R^2 of 99.52.

production. There are exciting times ahead as the next generation of NIR spectrometers will continue to improve as hardware and software become more advanced and the precision and accuracy of NIR technology steadily increase. One of biggest hurdles in implementing NIR methods is to generate reliable calibration models after the NIR instrument is purchased. In the foreseeable future, it is possible that instrument vendors or companies that specialize in NIR model development could provide 'turn-key' calibration for the distilling industry, leaving more time for distillers to focus on optimization of operations. NIR spectroscopy is here to stay; and companies that do not utilize this technology will run the risk of being left behind.

References

ASTM. 1997 Annual Book of ASTM Standards. Standard Practices for Infrared, Multivariate, Quantitative Analysis. Vol. 03.06. 827–852.

EMEA 2003. Note for Guidance on the Use of NIR Spectroscopy by the Pharmaceutical Industry and the Data Requirements for New Submissions and Variations. European Agency for the Evaluation of Medicinal Products, London. UK.

Griffiths, P.R. and J.A. de Haseth. 1986. *Fourier Transform Infrared Spectrometry*. John Wiley & Sons, New York.

Haaland, D.M., and E.V. Thomas. 1988. Partial least squares methods for spectral analyses. 1. Relation to other quantitative calibration methods and the extraction of qualitative information. Anal. Chem. 60: 1193 - 1202.

Herschel, F.W., Philos., Trans. R. Soc. London. 1800. 90:284-292.

Martens, H., and T. Næs. 1989. *Multivariate Calibration*. John Wiley & Sons Ltd. Chichester, UK

Williams, P.C. and K. Norris. 2001. Variables affecting near-infrared spectroscopic analysis. In: *Near-infrared technology in the agricultural and food industries. 2nd ed.* American Association of Cereal Chemists. 171 - 199.

Williams, P.C. 2001. Implementation of near-infrared technology. In: *Near-infrared technology in the agricultural and food industries. 2nd ed.* American Association of Cereal Chemists. 153-170.

Chapter 13

Emerging biorefineries and biotechnological applications of nonconventional yeast: now and in the future

CHARLES A. ABBAS

Director of Yeast and Renewables Research, Archer Daniels Midland, Decatur, Illinois, USA

Introduction

The biotechnology, food, and pharmaceutical industries have had a long and profitable association with yeast other than *Saccharomyces* (Abbas, 2001; Kurtzman *et al.*, 2001; Demain *et al.*, 1998; Fleet, 2000; Phaff, 1985). However in contrast to the widely utilized and studied members of the genus *Saccharomyces*, study of nonsaccharomyces yeast, frequently referred to as nonconventional yeast (NCY), has lagged behind – as has recognition of the vital role they play in ecological, biotechnological, and industrial applications. NCY have found many uses in the production of enzymes, polysaccharides, feed additives, fuels, flavors, chemicals, and most recently pharmaceutical and nutraceutical compounds (Table 1). The rapid growth in the use of NCY in industrial and biotechnological applications has been aided by continued advances in genetic engineering and from new developments in biochemical and physiological analyses. These applications exploit the wealth of the metabolic repertoire that this group of yeast possesses as well as their unique physiological adaptation to growth conditions and habitats that are challenging to *Saccharomyces*. This chapter aims to introduce several NCY of current or potential interest and encourage the reader to consider the variety of products possible in the distillery.

Taxonomic considerations

Since the taxonomic designations of yeast including that of NCY are forever changing, the reader is directed and encouraged to consult the two recent yeast taxonomic guides by Kurtzman and Fell (1998) and by Barnett *et al.* (2000). In the introduction to the guide by Barnett *et al.* (2000) the authors point out the confusion and many frustrations to which the discipline of yeast taxonomy lends itself. This is further illustrated by recent results of comparative genomic analyses that undoubtedly will force a revisitation of many of the current taxonomic designations. In spite of this, the above two guides provide a good current cross-reference for the majority of the currently recognized species of NCY that will be discussed. In the early chapters of these two guides the reader is provided with many of the tools employed today by modern taxonomists including the recent greater reliance on molecular and evolutionary approaches to define new families, orders, genera and species. This is not to say that taxonomists have abandoned morphological characteristics, reproductive cycles, physiological, ecological, and biochemical profiles as these continue to play a significant role in establishing yeast relationships and thereby their nomenclature. The role of morphological characterization and differentiation in nonconventional ascomycetous

Table 1. Selected current and potential biotechnological and industrial products derived from nonconventional yeast.

Amino acids	L-cystine, L-glutamic acid, L-serine, L-lysine, L-threonine, D and L-methionine; L-tryptophan, N-acetyl-glycine, L-phenylalanine
Carotenoids	Astaxanthin, carotene, lycopene
Chemicals	Acetoin, adipic acid, acetanilide, dicarboxylic acids, 1,3-propanediol, ethylacetate, 2,3-butanediol, glycerol, 1-ß-hydoxybutyric acid, α-ketoglutaric acid, lactic acid
Enzymes	Invertase, galactosidase, lipase, phytase, pectinases, trehalase, inulinase, glucoamylase, proteases, esterases
Feed additives	SCP, SCO, phytase, ß-glucans, riboflavin, amino acids
Fermented foods	Bread doughs, miso, soy sauce, ethanol
Flavors	2-phenlyethanol, nucleotides, ethylacetate, acetaldehyde, citric acid, furanones, methyl ketones
Food ingredients and additives	Citric acid, acetic acid, lactic acid, ribonucleotides, biosurfactants-sophorolids
Heterologous and other proteins	Enzymes, pharmaceuticals-vaccines, immunoproteins
Organic acids	Acetic, lactic, citric, iso-citric, formic, pimelic, homogentisic acids
Nutraceuticals, pharmaceuticals and cosmetics	N-acetylglucosamine, inositol, S-adenosyl methionine, melanin, tumor necrosis factor, human serum factors, ß-glucans
Polyols and sweeteners	Erythitol, glycerol, xylitol, D-arabitol, L-arabitol, D-mannitol
Simple sugars and polysaccharides	Xylulose, D-psicose, D-lyxose, mannans, phosphomannans, phosphogalactans, pullulans, mannoproteins, glucans, heteroxylans, exo-and acidic polysaccharides
Vitamins	Riboflavin (B_2), B_{12}, thiamin, ergosterol, ubiquinone

yeast consists of asexual vegetative reproductive growth and sexual reproduction as illustrated in Figures 1-3. In Figure 1, obtained with light microscopy, the use of budding and pseudohyphae with blastoconidia for the characterization and differentiation of several new isolates of Candida is provided (Kurtzman, 2001) and in Figure 2 a phase contrast microscopic photo of the commonly observed three cell division types, which consist of multilateral budding, bipolar budding, and fission in three different genera of yeast is depicted (Kutrzman *et al.*, 2001). Sexual reproduction or ascosporulation can be readily demonstrated for some species of ascomycetous NCY but with great difficulty for others. The ascospores formed exhibit a great variety of shapes as shown in Figure 3. In this chapter many of the most common names of NCY and their synonyms as designated by Barnett *et al.* (2000) will be used so the reader is encouraged when appropriate to consult with this source for additional information.

Ecological distribution of nonconventional yeast

The ecological distribution and natural habitats of NCY are diverse. NCY have been found world wide in association with decaying matter and in soil, fruits, cereal grains, dairy products, animal by-products, insects, bats, birds, as well as in numerous plant and animal-derived products such as syrups, cheeses, alcoholic beverages, and other fermented foods (Fleet, 2000). Yeast ecologists and taxonomists recognized early on the significance of the distribution of NCY in their natural habitats and their ability to grow on a variety of substrates under various and occasionally extreme conditions. This recognition paved the way for the exploitation of NCY initially in food production and later further expanded to a variety of industrial applications through use of strain development and selection programs. In the excellent chapter by Lachance and Starmer (1998), the authors

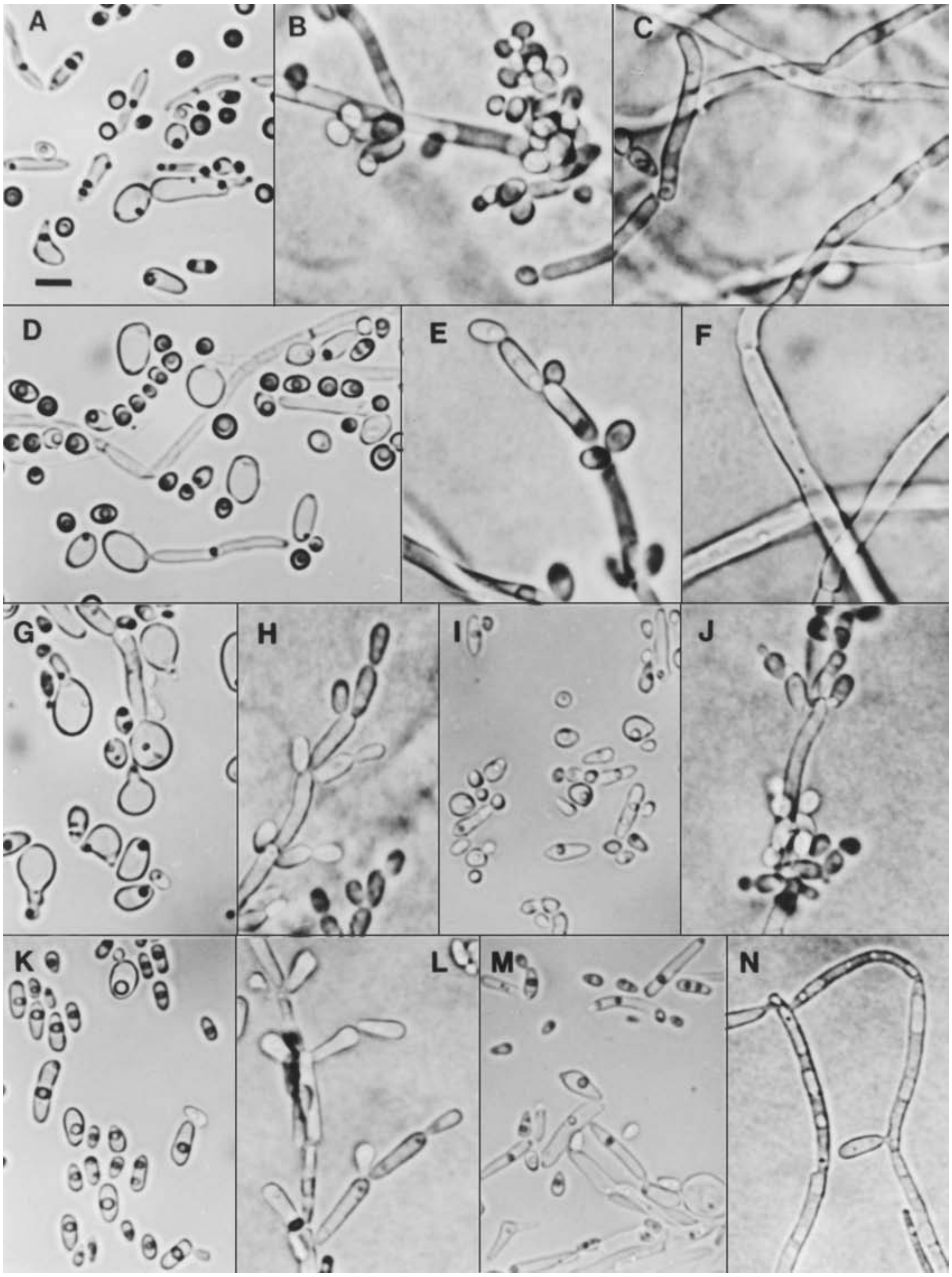


Figure 1. Morphological differentiation and characterization of six new species of *Candida*. Budding cells are depicted in A, D, G, I, K,and M. Pseudohyphae with blastoconidia are depicted in B, C, E, F, H, J, L and N. Budding cells are from 5% malt extract agar after 3 days at 25°C while pseudohyphae are from Dalmau plate cultures on yeast morphology agar following 7 days of incubation at 25°C. Light microscopic photo was provided courtesy of Dr. Cletus Kurtzman.

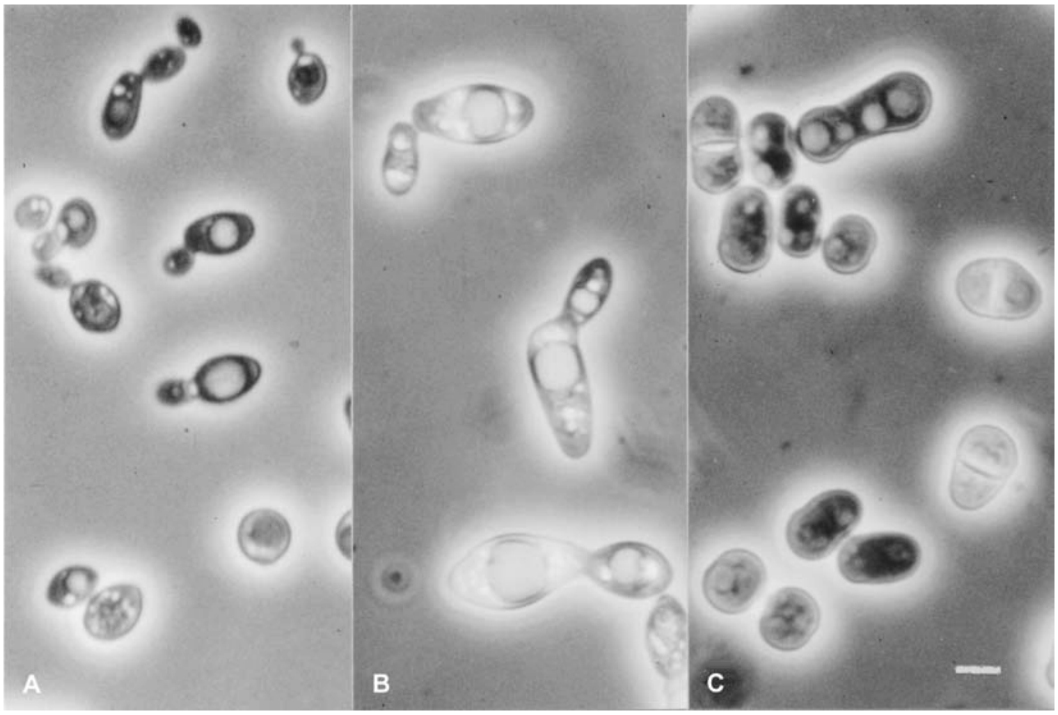


Figure 2. Cell division in the ascomycetous yeast. A) Multilateral budding in *Saccharomyces cerevisiae;* B) Bipolar budding in *Saccharomycodes ludwigii*; C) Fission in *Schizosaccharomyces octosporus*. Phase contrast photo was provided by Dr. Cletus Kurtzman.

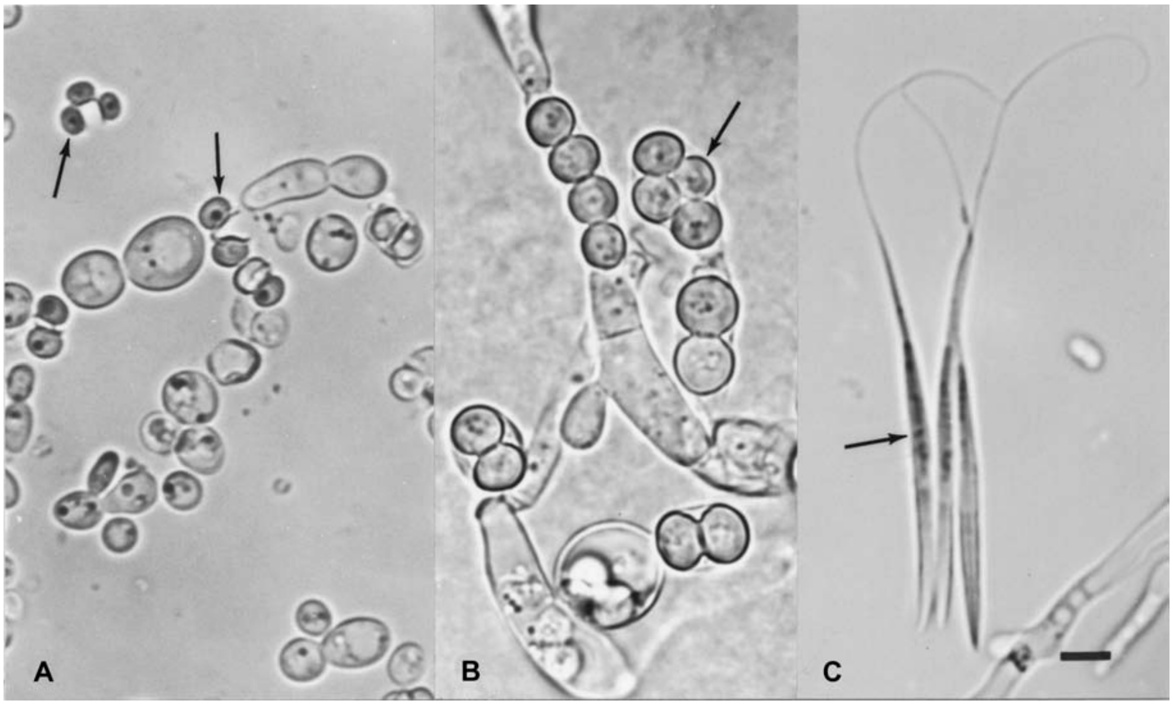


Figure 3. Representative ascospores of A) Hat-shaped (galeate), *Pichia bispora*; B) Spheroidal, *Saccharomycopsis capsularis*; C) Elongate with a whip-like extension of the cell wall, *Eremothecium (Nematospora) coryli*. Bar=5 microns. Bright field photomicroscopic photo provided by Dr. Cletus Kurtzman.

stress the significant contribution of yeast biology to the study of evolutionary ecology and the valuable role played by yeast in the interaction of the microbial communities in the ecosystem. As such, one cannot ignore the role of yeast in climatic changes and the need for the research community to utilize multidisciplinary approaches to bridge the gaps in the fundamental knowledge of the significance of yeast to ecological studies.

NCY industrial strain development programs

Historically strain development programs with NCY have followed the same path successfully taken with the *yeast Saccharomyces cerevisiae* (Benitez *et al.*, 1996; Penttila, 1997; Beckerich *et al.*, 1982; Phaff, 1985). These approaches have often consisted of classical mutagenesis followed by screening and selection, yeast hybridization using mating followed by sporulation and tetrad analysis (ploidy dependent) or rare mating (ploidy independent), and the employment of parasexual genetics using protoplast fusions (Phaff, 1985; Panchal *et al.,* 1984; Beckerich *et al.,* 1982; Benitez *et al.*, 1996). The advent of molecular biology gave rise to the use of new approaches in NCY strain development with increased use of recombinant DNA to transform yeast via incorporation of genes on plasmids acting as shuttle vectors or by direct chromosomal integration. The development of tools for the genetic manipulation of NCY is described in greater detail in several recent texts that cover progress in this area (deWinde, 2003; Wolf, 1996; Wolf *et al.*, 2003). Using the listed approaches in this section numerous homologous and heterologous genes have been cloned and successfully expressed in most NCY of industrial importance as well as those of current interest (deWinde, 2003; Wolf, 1996; Wolf *et al.*, 2003). As newer yeast strain development approaches are increasingly more reliant on genomic and metabolic engineering analyses, these approaches are also emerging as new tools with NCY as well. The combination of genome sequencing with gene expression profiling using microarray chips, 2D protein electrophoresis, and biochemical and metabolic flux analyses has been adopted as part of strain development strategies to improve NCY strains for commercial applications. While the application of these approaches in NCY lags behind that of *S. cerevisiae*, great progress in this area has been facilitated by the major effort to sequence a number of NCY of industrial and biotechnological importance as a part of the Genolevures undertaking in France. This impressive sequencing program has sought to delineate and compare the functional genomes of hemiascomycetous yeast using random sequence tags (RST) to obtain the maximum biologically relevant data with a minimum of effort. The selected list of 13 ascomycetous yeast included several NCY yeast of industrial and biotechnological significance (Figure 4). A detailed and a complete description of the results of the initial phase of this effort was published in FEBS Letters in December of 2000 and a recent update of the latest progress was presented at Yeast 2003 in Göteborg, Sweden (Dujon, 2003).

Yeast physiology and biochemistry

NCY yeast physiology and biochemistry continue to be active and attractive areas of research (deWinde, 2003; Wolf, 1996; Wolf *et al.*, 2003). The greater recent attention to NCY has resulted from great strides made in our understanding of the genetics, physiology and biochemistry of the yeast *S. cerevisiae*. In *S. cerevisiae* this has given rise to what is currently referred to as the discipline of system biology. System biology seeks to integrate knowledge gained from genetic, biochemical, structural, and physiological research to develop a comprehensive approach to our understanding of the metabolism of yeast and other organisms (Nielsen and Olsson, 2002). An essential component of this approach is to provide a detailed description of the interactions among all components within a cell. In order to pave the way for the use of this approach to NCY, research must address many of the areas listed in Table 2. Due to space limitations the reader is urged to consult with many of the excellent references provided at the end of this chapter. The successful application of this approach to

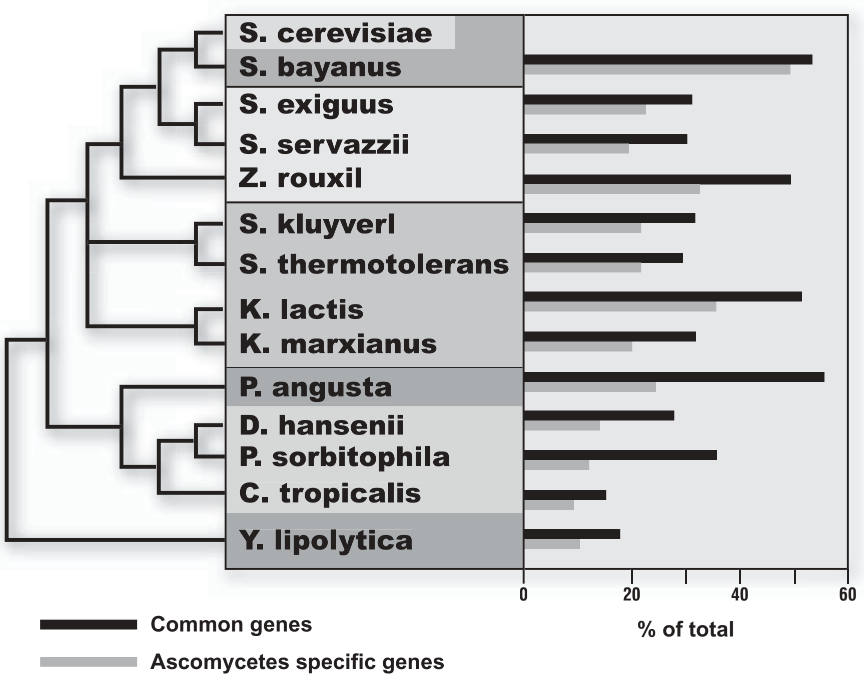


Figure 4. Genolevures: Comparative genomics of 13 hemiascomycetous yeast.

NCY will undoubtedly have a significant impact on the increase in industrial and biotechnological uses of these yeast.

Table 2. List of biochemical, cellular, metabolic, nutritional and physiological topics currently studied in yeast.

Cell wall chemistry, organization, and flocculation
Growth kinetics
Nutritional requirements and metabolism of nutrients
Regulation of C/N metabolism
Stress responses and signal transduction
Sugar utilization profiles
Sugar transport and energetics
Protein synthesis, trafficking, secretion and turnover
Yeast organelles biogenesis and function
Yeast cell structure, compartmentalization, and organization
Yeast cell cycle, control of growth and cellular division
Yeast aging and apoptosis
Genomics: structural, functional, comparative, evolutionary

NCY of biotechnological importance

CANDIDA UTILIS (ASEXUAL STATE OF *PICHIA JADINII*)

Candida utilis (also known as *Torula utilis* or Torula yeast) has been isolated from human and animal sources and from distillery and yeast factories (Phaff, 1985; Kurtzman and Fell, 1998; Barnett *et al.,* 2000). Vegetative reproduction in this yeast occurs by budding with filaments or simple pseudohyphae occasionally reported. Sexual reproduction has been recognized; and as a result of this its relatedness to *Pichia jadinii* accepted by taxonomists. Due to some concerns about the potential pathogenicity of this yeast it has been classified at a biological safety level I (Barnett, 2000). This is in spite of the long safety record attributed to this yeast and its acceptance by the US FDA as a safe substance and its approval as a food additive (Miura *et al*., 1999).

C. utilis has been reported to produce a variety of products ranging from amino acids (L-serine; D-methionine; L-glutamic acid), glutathione, S-adenosyl methionine, RNA (9-ß-D-ribofuarnoside-5´-phosphoric acid esters of 2-substituted-6-hydroxypurine), coenzyme A, single cell proteins (SCP), polyols (xylitol), organic acids (acetic acid, adipic acid), sophorolipids, ethylacetate, methyl ketone, ethanol and acetaldehyde. Most recently this yeast has been genetically engineered to produce a number of carotenoids, which include lycopene, ß-carotene and astaxanthin as well as for the heterologous production of a number of proteins including the enzyme α-amylase and the sweet potato protein, monellin (Miura *et al.,* 1999; Miura *et al.,* 1998; Shimada *et al.,* 1998). A number of enzymes of this yeast have been isolated and characterized. These include alcohol dehydrogenase, allantoin racemase, 2,3-butanediol dehydrogenase, dimethylamine mono-oxygenase, dihydroxyacetone reductase, glutamine synthetase, invertase, maltose permease, trimethylamine mono-oxygenase, trehalase, urate oxidase, and urease just to name a few. *C. utilis* is known to assimilate inexpensive sugars produced by the hydrolysis of lignocellulose feedstocks such as alfalfa wastes, cellulose, hemicellulose, pineapple waste, potato waste, and sugarcane bagasse. It can also utilize and degrade petroleum waste, rapeseed oil meal, sauerkraut waste streams and tallow. Since *C. utilis,* unlike *S. cerevisiae,* cannot produce ethanol under strict aerobic conditions, it can be grown in continuous culture to a high density (Miura *et al.,* 1999). These are desirable features that have been exploited in SCP, in the production of extracellular enzymes and other proteins as well as in the production of intracellular proteins and other metabolites such as carotenoids by this yeast. A recent taxonomic description of this yeast can be found in Barnett *et al.* (2000) on pages 532-533.

CANDIDA TROPICALIS

Candida tropicalis is found in association with human beings, soil, water, flowers, and in foods such as kefir, miso, dates, sauerkrauts, and other fermented foods produced by mixed culture fermentations. This yeast has also been recovered as a contaminant from vegetable oil processing facilities, blackstrap molasses, rotten pineapples, and from citric acid fermentations. Vegetative growth in this yeast occurs by budding with elaborate or septate hyphal formation. No sexual reproductive cycle is known for this yeast. *C. tropicalis* has been reported to produce SCP, ergosterol, xylitol, several amino acids (L-cystine, L-trptophan; L-methionine; N-acetyl glycine), acetanilide, adipic acid, carnitine, ethanol, glutaric acid, L-iditol, mannans, ribonucleic acids, and ubiqinone. Tanaka *et al.* (1971) recognized the ability of *C. tropicalis* to grow on n-alkanes and most recently this yeast has been genetically engineered to efficiently produce long-chain dicarboxylic acids from alkanes and fatty acids (Fabritius *et al.*, 1996; Picataggio *et al.*, 1992). These acids are of commercial interest as they can serve as precursors of polyamides, fragrances, and polyhydroxyalkenoates (Fabritius *et al.*, 1996). *C. tropicalis* has also been widely studied for the production of xylitol from xylose derived from lignocellulosics, where it has shown great promise in achieving high rates of conversion, high yield, and high concentration in pilot plant studies (Yahashi *et al.,* 1996; Choi *et al.*, 2000; Kim *et al.*, 1999; Oh and Kim, 1998). A number of enzymes from this yeast have been isolated and characterized including alcohol oxidase, carnitine acetyl transferase, catalase, and urate oxidase. *C. utilis* has been shown to utilize and/or degrade hydrocarbons, kraft black liquor from phenol, D-xylose, petroleum, and paper mill waste. The inclusion of this yeast in the initial Genolevures comparative genomic exploration is described by Blandin *et al.* (2000a). A detailed update taxonomic description is found in Barnett *et al.* (2000) on pages 270-271.

CANDIDA FAMATA (ASEXUAL STATE OF *DEBAROMYCES HANSENII*)

Candida famata is among the most widely distributed of the NCY. This yeast has been isolated from a wide variety of fermented foods and beverages as well as from human and animal sources. Vegetative reproduction is by budding with filaments and simple pseudohyphae formed. Persistent asci are formed by this yeast that contain 1-2 rough, round ascospores (Barnett *et al.*, 2000). Recent taxonomic guides list this yeast as the asexual state of *Debaromyces hansenii*. The production of the polyols D-arabitol and xylitol, SCP, the organic acids citric and isocitric acid, production of D-lyxose from D-glucose, production of D-talitol from D-psicose, production of antimicrobials, and the vitamin riboflavin (B_2) have all been reported (Lim *et al.*, 2001; Ahmed *et al.*, 1999; Stahmann *et al.*, 2000; Sasahara *et al.*, 1998; Linardi *et al.*, 1996). Special features of this yeast include its osmotolerance as indicated by its high glucose and salt tolerances as well as its ability to grow on hydrocarbons and the pentose sugars D-xylose and L-arabinose that are present in hydrolysates of lignocellulosics (Roostita and Fleet, 1999). Flocculation has been described for this yeast in the presence of peptone and its involvement in the formation of biofilms has been noted (Martinez *et al*., 1996). Recently a transformation system has been developed and optimized for this yeast (Voronovsky *et al*., 2002) and the use of PCR to identify and distinguish this yeast from other *Candida* has been published (Nishikawa *et al*., 1997; 1999). Due to the inclusion of *D. hansenii* in the Genolevures comparative genomic project, the genomic sequence of this yeast has been delineated and compared to a number of other ascomycetous yeast (Lepingle *et al*., 2000; Dujon, 2003). Recently, attempts to characterize and isolate genes responsible for salt tolerance in *D. hansenii* have been reported (Ramos *et al*., 2003). Understanding the underlying mechanism for salt tolerance in this yeast can have a significant impact on its further commercial exploitation as it has been used for over a decade for the large-scale commercial fermentation production of the vitamin riboflavin (B_2) (Abbas, 2001). A complete recent description of *D. hansensii* is provided by Barnett *et al.* (2000) on pages 336-337.

YARROWIA LIPOLYTICA (ASEXUAL STATE OF *CANDIDA LIPOLYTICA*)

Yarrowia lipolytica is frequently found in association with dairy and with waste streams derived from vegetable oil processing facilities. Reproduction primarily occurs via budding and filaments of pseudohyphae are frequently observed microscopically. Sexual spores are formed that resemble rough, oval, hat-, Saturn, or walnut-shaped ascospores, and have been reported following mixing of sexually compatible strains. Recent taxonomic guides have recognized that *C. lipolytica* is the asexual state of *Y. lipolytica* (Barnett *et al*., 2000). The production of organic acids such as citric, 2-methyl isocitric, isocitric acid, homogentisic (breakdown products from metabolism of L-phenylalanine), pimelic acid, α-ketoglutaric acid and L-ß–hydroxybutyric acid, the polyols mannitol, xylitol and erythritol, lipases active at pH 5.5 and 7.5, lipases active at pH 9.0 and 60°C, lipase activators, proteases, alkaline protease, melanin, and single cell protein have all been reported for this yeast (Kyong and Shin, 2000; Delgenes *et al*., 1998; Silva *et al*., 1996; Rodrigues *et al*., 1998). One of the many interesting characteristics of this yeast is the ability to hydrolyze and reduce zearalenone, as well as the ability to degrade other aromatic compounds, fatty acids, and alkanes such as azelaic acid, benzo (α) pyrene, biphenyls, hydrocarbons, naphthalene, petroleum, rapeseed oil, and tallow (Hassan anbd El-Sharkway, 1999; De Felice *et al*., 1997). The above properties have been exploited in the production of organic acids and enzymes using ethanol, glucose, fatty acids, or hydrocarbons as the primary carbon substrates (Arzumanov *et al*., 2000; Antonucci *et al*., 2001; Wache *et al*., 2003; Pazouki *et al*., 2000). More recently the development of an efficient transformation system for *Y. lipolytica* has aided further in the expansion of the uses of this yeast to the production of proteins such as enzymes and pharmaceuticals (Chang *et al*., 1998; Barth and Gaillardin, 1996). The inclusion of this yeast in the Genolevures 1 and 2 comparative genomics project has aided in its genomic analysis and comparison to other hemiascomycetous yeast (Dujon, 2003; Casaregola *et al*., 2000). A complete recent taxonomic description is provided by Barnett *et al*. (2000) on pages 785-786.

CANDIDA GUILLIERMONDII (ASEXUAL STATE OF *PICHIA GUILLIERMONDII*)

Candida guilliermondii is commonly found in association with human beings, animal products and by-products as well as in soil, water, dairy products, dough, insects, and fruit juices. Vegetative reproduction in this yeast occurs by budding and filaments. Either none or elaborate pseudohyphae are observed by microscopy. Four hat-shaped ascospores are formed by conjugation of sexually compatible strains upon mixing. When cultured on corn meal agar for 11 days, extensive and long filaments have been reported. *C. guilliermondii* is a flavinogenic yeast that accumulates riboflavin intracellularly. Other products produced by this yeast are SCP, ethanol, citric acid, xylitol from D-xylose, L-arabitol from L-arabinose, as well as tea cider (Saha and Bothast, 1996). Two enzymes that have been isolated and well characterized from this yeast are alcohol dehydrogenase and deaminase. Due to the interest in the production of xylitol by *C. guilliermondii,* the impact of acetic acid and sugar degradation products present in a number of lignocellulosic hydrolysates from the two enzymes involved in D-xylose metabolism in this yeast, xylose reductase and xylitol reducatase, has been extensively studied. Growth of this yeast in brine and on cellulose hydrolysates has been reported. The recent use of this yeast as a biocontrol agent to reduce postharvest fruit spoilage has been described. Genetic transformation of *C. guilliermondii* has been reported (Boretsky *et al.*, 1999) and a detailed description of the genetics of this yeast provided by Sibirny (1996a). A complete description of the taxonomy of this yeast is provided in Barnett *et al.* (2000) on pages 523-524.

KLUYVEROMYCES LACTIS

Kluyveromyces lactis is another milk-sugar fermenting yeast that has been isolated from a wide range of sources, which include dairy factories, wineries, human sources, and plant sources such as the slime of an oak tree. This yeast been shown to produce ethanol from whey produced from cheese manufacturing. Some strains of this NCY are known to harbor yeast protein killer factors. Genetic studies of this yeast have led to the development of efficient transformation systems for the production of proteins such as the enzyme ß-galactosidase and the proteinase calf chymosin, as well as many other proteins that are listed in the extensive review by Wesolowski-Louvel *et al.* (1996). Most of these proteins are commercially important in pharmaceutical applications. The metabolism of galactose, high cell density cultivation, the pattern of N-protein glycosylation, and its codon usage choice, which is similar to that of *S. cerevisiae,* have been well studied (Wesolowski-Louvel *et al.*, 1996). Another recent development with this NCY is its inclusion in the Genolevure comparative sequencing efforts, which has provided further information to support its relatedness to the other hemiascomycetous yeast, *K. marxianus* (Bolotin-Fukuhara *et al.*, 2000; Dujon, 2003). The taxonomic description of this yeast has been described by Barnett *et al.* (2000) on pp. 417-418.

KLUYVEROMYCES MARXIANUS (SYNONYM *K. FRAGILIS* AND ASEXUAL STATE *CANDIDA KEFYR*)

K. marxianus is often associated with spoilage of commercial yogurt and has been commonly isolated from other dairy products such as milk and cheese, pressed commercial yeast, effluent of sugar refineries, bread doughs, beer, fermenting figs, sewage, infected humans and infected milk cows, where it causes bovine mastitis. In spite of the association of *K. marxianus* with infected humans and dairy cows, it is considered GRAS and frequently referred to as the 'dairy yeast'. Vegetative reproduction in this yeast occurs via budding; and simple to elaborate pseudohyphae are produced. Due to the production of ascospores, *K. marxianus* is currently classified as the perfect state of *C. kefyr,* while the closely related *K. fragilis* is listed as one of its many synonyms (Barnett *et al.*, 2000). When cultured on corn meal or potato dextrose agar for 8-11 days, long filaments are observed microscopically. *K. marxianus* has been reported to produce ethanol from cheese whey, oligonucleotides for use as flavor enhancers, polyphosphates, metallothionein, glycerol, single cell protein from biomass, several enzymes for commercial application (lactase and pectinases), pectin extraction from fruits, polyphosphate, and glycerol (Parrondo *et al.*, 2000; Ferarri *et al.*, 1996; Belem *et al.*, 1997; Belem *et al.*, 1998). In addition to lactases (ß-galactosidase) and pectinases, several other enzymes have been isolated, characterized, and cloned including inulinase, alcohol dehydrogenase, acetoin dehydrogenase, and UDP glucose-4-epimerase (Schwan *et al.*, 1997). Interesting characteristics of this yeast include the production of killer factors and growth and production of ethanol from cheese whey and inulin at 45°C to a final concentration of 7.0% (w/v) (Balletsteros *et al.*, 1993; Singh *et al.*, 1998; Banat *et al.*, 1995). *K. marxianus* can grow on a wide range of carbon sources and waste streams such as lactic acid, sauerkraut waste, orange peel, paper mill sludge, and cocoa pulp. A simultaneous saccharification and fermentation process to produce ethanol that combines fungal cellulase enzymes with this yeast has been reported (Lark *et al*., 1997). The results have indicated that further development with the genetic engineering of *K. marixanus* to ferment lignocellulosics to ethanol is feasible and attractive given this yeast's ability to grow on a variety of feedstocks and its thermotolerance. *K. marxianus* has been shown to accumulate toxic compounds including cadmium, copper, and silver, and has also been used in adhesive testing. The controversy over the relatedness of this yeast to *K. lactis* is cited by Wesolowski-Louvel *et al*. (1996) and Barnett lists *K. lactis* and *K. marxianus* separately as several lines of evidence clearly distinguish between these two yeasts. Both of these yeasts were analyzed as a part of the Genolevures comparative genomic sequencing and results reported (Bolotin-Fukuhara *et al*., 2000; Llorente *et al*., 2000). A complete description of this NCY is provided in Barnett *et al*. (2000) on pages 420-421.

CRYPTOCOCCUS LAURENTII

Cryptococcus laurentii is commonly associated with cereal grains, grapes, soil, birds, decaying plant matter, air, seawater, and tropical plants. Vegetative reproduction occurs by budding and filaments are present or absent with the formation of simple and/or septate hyphae. Sexual reproduction in this yeast has not been reported. *C. laurentii* is known to produce a number of exo- and acidic polysaccharides, xylanases and glycosidases, a thermostable glutaminase, killer toxins, L-lysine, and a number of diol compounds. Other characteristics of this yeast include its alkali tolerance and its ability to degrade benzene derivatives. Due to *C. laurentii's* ability to produce copious levels of extracellular polysaccharides, there has been great interest in developing food and non-food applications for their use (De Baets and Vandamme, 1999; Nadezda *et al*., 1997). Another area that has received commercial interest is use of this yeast as a biocontrol agent to retard fruit spoilage (Giuseppe *et al*., 1998; Castoria *et al*., 1997). The involvement of 1,3-ß-glucanases as the mode of action against spoilage fungi has been implicated as the protective mechanism. A complete description of this yeast is provided by Barnett *et al*. (2000) on pages 315-316.

ZYGOSACCHAROMYCES ROUXII

Zygosaccharomyces rouxii is one of the most common food and beverage spoilage yeast. Vegetative reproduction of this yeast occurs via budding with the presence or absence of filaments or simple pseudohyphae. Persistent asci formation containing up to four rough or smooth sound ascospores has been noted for this yeast. Among the many products of this yeast are acetic acid, formic acid, ethanol, alkyl esters, L-glutamine, mannans, miso, 4-hydroxyfuranone, soy sauce, lipases that are active at pH 9.0 at 60°C, UDP-N-acetylglucosamine, and 5´-nucleotides (Hayashida *et al*., 1998). Important characteristics of this yeast are its resistance to high salt, sugar, sorbic acid, and tolerance to high pressure, which contribute to its survival in and spoilage of preserved foods and beverages. Due to this property, this yeast is increasingly being targeted for biotechnological applications (Limtong *et al*., 1998). The inclusion in the Genolevures comparative genomics effort will propel further rapid developments in the genetic manipulation and exploitation of *Z. rouxii* (De Montigy *et al*., 2000; Sychrova *et al*., 2000). The isolation of ura3 mutants and the development of an efficient transformation system were recently described by Pribylova and Sychrova (2003). A complete description of the taxonomy of this yeast is provided in Barnett *et al*. (2000) on page 797.

CANDIDA BOIDINII

Candida boidinii have been isolated from a wide range of geographic locations from a number of sources such as tanning solutions, fermented acidic products, swamps and other water habitats and flowers. Vegetative reproduction occurs by budding with the formation of simple to elaborate pseudohyphae. No sexual reproduction stage has been described for this yeast. *C. boidinii* grows on and degrades methanol as well as pectin and can produce a number of products of interest such as the polyols xylitol and L-arabitol, xylulose, SCP, nucleotides such as ATP, ethanol, D-amino acids, as well as a number of pigments (Vandeska *et al*., 1996; Suryadi *et al*., 2000; Yamada *et al*., 2001). Several enzymes have been isolated and characterized from this yeast including NAD-linked alcohol dehydrogenase, polyamine oxidase, formate dehydrogenase, diamino acetyltransferase, dihydroxyacetone synthase, and S-formylglutathione hydrolyase (Labrou *et al*., 2001). Due to *C. boidinii's* ability to grow on methanol, the metabolism of this carbon source by this yeast has been extensively studied (Yurimoto *et al*., 2000, Sakai *et al*., 1998; Gabrinska-Loniewska, 1997; Aggelis *et al*., 1999). The development of genetic tools and a transformation system have enabled the cloning and expression of a number of heterologous proteins in this yeast (Sakai *et al*., 1996; Nakamura *et al*., 2000). Significant interest has also been shown in the use of this yeast for the commercial production of dicarboxylic acids. A recent taxonomic description of *C. boidinii* can be found in Barnett *et al*. (2000) on page 145.

PICHIA PASTORIS

Pichia pastoris can be found in exudates of hardwood trees such as black oak and chestnut. Vegetative reproduction in this yeast is by budding and no filaments are formed. The formation of asci containing up to 4 hat-shaped ascospores have been noted in this yeast (Barnett *et al*., 2000). *P. pastoris* has attracted much interest in biotechnological applications that involve heterologous proteins. Its ability to grow on methanol as a carbon source has been extensively characterized and the peroxisomal system described in great detail. The development of genetic tools for this yeast has led to the rise in the use of commercial laboratory kits and the demonstration of high-level production of a number of pharmaceutical and other proteins by *P. pastoris*. In a properly designed fermentation process this yeast can be cultivated to high density, which is key to demonstrating commercial feasibility of processes that are dependent on optimizing cell mass production (Chung, 2000; D'Anjou and Dauglis, 2001; Thorpe *et al*., 1999; D'Anjou and Dauglis, 2000; Minning *et al*., 2001; Trujillo *et al*., 2001; Rodriguez *et al*., 1996; Sberna *et al*., 1996). In addition to its ability to grow on methanol and other oxygenated hydrocarbons, *P. pastoris* can degrade hydrogen peroxide and can be used to produce acetaldehyde and SCP. Due to space limitations, the reader is directed to excellent recent reviews of this yeast that describe in greater detail its biotechnological applications (Gellissen, 2000; Sreekrishna and Kropp, 1996). Comparative genomic sequence data comparison from inclusion of this yeast in Genolevures was reported (Blandin *et al*., 2000) A recent description of this yeast is provided by Barnett *et al.* (2000) on page 554.

PHAFFIA RHODOZYMA (ANAMORPH OF *XANTHOPHYLLOMYCES DENDRORHOUS*)

Phaffia rhodozyma is a carotenogenic yeast that was originally isolated from the stump of birch trees in Japan, Finland and Russia and from an alder tree in Japan. This yeast reproduces vegetatively by budding with formation of filaments with simple pseudohyphae present or absent. Sexual spores or basidiospores are borne on basidia. The current taxonomic classification of *P. rhodozyma* as an anamorph of *X. dendrorhous* has not been accepted by Kurtzman and Fell (1998). Due to its ability to produce the carotenoid astaxanthin, which is a valuable additive in salmon diets, this yeast has been of important commercial interest. The availability of improved strains of *P. rhodozyma* that can economically produce astaxanthin intracellularly has aided in the development of industrial scale high cell density fermentations (Abbas, 2001). The production of other carotenoids by *P. rhodozyma* has also been reported. Cultivation of this yeast to produce astaxanthin from several nonconventional carbon sources such as wood hydrolysates, date juice, glycerol, D-xylose, a number of disaccharides, as well as on dried distillers grains produced from ethanol dry mill plants has also been noted (Vazguez *et al*., 1998; Parajo *et al*., 1998a,b; Fontana *et al*., 1997; Santopietro *et al*., 1998; Ramirez *et al*., 2000; Chan and Ho, 1999; Cruz and Parajo, 1998; Florencio *et al*., 1998; Kesava *et al*., 1998). A transformation system has also been developed for this yeast and improved recently (Wery *et al*., 1998; Visser, 2003). A detailed taxonomic description of this yeast is provided in Barnett *et al.* (2000) on page 784.

HANSENULA POLYMORPHA (SYNONYM *PICHIA ANGUSTA*)

Hansenula polymorphia has been isolated from a number of sources in different parts of the world, which include spoiled orange juice, maize meal, the fruit fly *Drosophila pseudoobscura*, milk from cows with mastitis, and soil irrigated with a distillery effluent. Vegetative reproduction occurs by budding and no filaments are formed. Asci that contain up to 4 hat-shaped ascospores have been described for this yeast. *H. polymorpha* is characterized by its excellent growth on short chain oxygenated hydrocarbons such as ethanol and methanol as well as other sugar alcohols or polyols, which include glycerol, erythritol, xylitol, and L-arabitol (Sanchez *et al*., 1998; Suryadi *et al*., 2000). Due to its growth on methanol, the physiology and biochemistry of this yeast has been well studied and the metabolism of this carbon source is well understood. Based on the ability of this yeast to grow on methanol and ethanol, alcohol biosensors have been described that utilize coupled oxidase-peroxidase enzymes (Vijayakumar *et al*., 1996). The development of genetic tools for *H. polymorpha* has given rise to the genetic manipulation of this yeast and the production of a number of heterologous proteins of commercial interest such as maltase, L-rhamnosidase, and phytase (Liiv *et al*., 2001; Mayer *et al*., 1999; Yanai and Sato, 2000). This has led to numerous biotechnological applications of this NCY and due to space limitations the reader is urged to consult with excellent recent reviews cited at the end of this chapter (Hansen and Hollenberg, 1996; Hollenberg *et al*., 1997; Gellissen, 2000). A detailed taxonomic description is provided in Barnett *et al.* (2000) on page 493.

SCHIZOSACCHAROMYCES POMBE (SYNONYM *SACCHAROMYCES POMBE*)

Schizosaccharomyces pombe was originally isolated from East African millet beer and since then from a variety of alcoholic beverages such as palm wine, arak, beer breweries, and from sulphited grape juice, grapes, cane sugar, and molasses. This yeast is fairly distinguishable using microscopy by its large size and by its vegetative reproduction by splitting or fission. The production of filaments in this yeast has not been observed. Sexual reproductive asci are produced with up to four smooth, oval, or round ascospores reported (Barnett *et al*., 2000). Due to its unique morphological, biochemical, physiological, and metabolic features, *S. pombe* has received much recent attention for biotechnological applications. These applications have taken advantage of its high osmotolerance, high temperature tolerance and the development of genetic tools for its exploitation (Dhamija *et al*., 1996; Chaudhari and Chincholkar, 1999). Among the many products that can be produced with *S. pombe* are ethanol, SCP, and heterologous proteins for use in food and pharmaceutical applications (Amanchukwu *et al*., 1989; Fukuda *et al*., 1984). An excellent recent text by Giga-Hama (1997) describes many of the heterologous proteins that can be produced by this yeast. A detailed taxonomic summary is provided by Barnett *et al.* (2000) on pages 678-679.

OTHER NONCONVENTIONAL YEAST OF INTEREST

In addition to the NCY described in detail in the previous section there are many others that are of current interest to the biotechnology industry. These include several that can produce ethanol from sugars present in lignocellulosic hydrolysates such as *Candida shehatae, Candida wickerhamii, Pichia stipitis, and Pachysolen tannophilus* (Sreenath and Jeffries, 1999; Sanchez *et al*., 1999; Amartey and Jeffries, 1996; Possoth *et al*., 1996; Kruse and Schuegerl, 1996; Skory *et al*., 1996; Freer *et al*., 1998). The amylolytic NCY *Debaromyces (Schwanniomyces) occidentalis, Lipomyces starkeyi* and *Aruxla adeninivorans* have been recently tapped for their ability to grow on starch and have other attributes that can be exploited further for a wide range of biotechnological applications (Kunze and Kunze, 1996; Wartmann and Kunze, 2000; Laires *et al*., 1983). The yeast *Candida bombicola, C. rugosa*, and *C. maltosa* can grow on n-alkanes, fatty acids, waste animal fat or waste vegetable oils to produce products such as single cell oils, biosurfactants, and 1,3-butanediol (Sharyshev *et al.*, 1997; Montesinos *et al.*, 2003; Matsuyama *et al.*, 2001; Deshpande and Daniels, 1995; Rau *et al.*, 1999; Daniel *et al.*, 1998; Rau *et al.*, 2001; Lang *et al.*, 1996; Daniel *et al.*, 1999; Mauersberger *et al.*, 1996). The NCY *Candida krusii, Candida magnolia* and *Pichia farinosa* can produce polyols such as glycerol and erythritol (Abbas, unpublished; Sung-Wook *et al.*, 1999; Nakano *et al.*, 2000; Yamazaki *et al.*, 2000). The methylotrophic yeast *Pichia methanolica* can grow on methanol (Sibirny, 1996). The yeast *Pichia fermentans* can produce the flavoring compound, 2-phenylethanol (Huang *et al.*, 2000). Several NCY, which include *Rhodotorula aurantiaca, R. rubra*, and *R. glutinis* have been evaluated as biocontrol agents to prevent fruit harvest decay while the yeast *Pichia anomala* has been shown to inhibit mold growth during cereal grain storage (Chand-Goyal *et al.*, 1996; Sugar and Spotts, 1999; Lima *et al.*, 1998). *Rhodotorula glutinis* and *R. rubra* are also known for their production of the carotenoids ß-carotenes and lycopene (Bhosale and Gadre, 2001; Matelli *et al.*, 1990; Buzzini, 2000; 2001). Several NCY are well known pathogens such as *Candida albicans* and *Filobasidiella (Cryptococcus) neoformans* and therefore have received for many decades much attention from the medical and the pharmaceutical industries (Demain *et al.*, 1998). Finally, many NCY have been recognized for their role in food spoilage. Some of the many NCY that are involved in food spoilage are members of the genera *Brettanomyces, Pichia, Candida, Torulaspora, Metschnikowia,* and *Hansenisapora* (Grbin and Henschke, 2000).

Future predictions and conclusions

Over the next decade the commercial value of all products derived from the biotechnological applications of NCY will surpass all those derived from the current uses of food/fuel/feed/chemical as well as pharmaceutical and nutraceutical uses of *S. cerevisiae* (Abbas, 2001). Rapid advances in metabolic engineering in combination with bioinformatics and improved fermentation processes from the greater employment of metabolic controls aided by increased automation will fuel much of the progress in the uses of NCY in industrial and biotechnological applications. With these developments in mind, the next decade promises to be an exciting era for the biotechnological and industrial uses of NCY.

References

Abbas, C.A. 2001. Industrial applications of nonconventional yeast. Plenary presentation abstract, ISSY 2001, Lviv, Ukraine.

Aggelis, G., S. Fakas, S. Melissis and Y.D. Clonis. 1999. Growth of *Candida boidinii* in a methanol-limited continuous culture and the formation of methanol-degrading enzymes. J. Biotech. 72(1/2):127-139.

Ahmed, Z., H. Sasahara, S.H. Bhuiyan, T. Saiki, T. Shimonishi, G. Takada and K. Izumori. 1999. Production of D-lyxose from D-glucose by microbial and enzymatic reactions. J. Bioscience and Bioeng. 88(6):676-678.

Amanchukwu, S.C., A. Obafemi and G.C. Okpokwasili. 1989. Single-cell protein production by *Schizosaccharomyces pombe* isolated from palm wine using hydrocarbon feedstocks. Folia Microbiol. (Prague) 34(2):112-119.

Amartey, S. and T. Jeffries. 1996. An improvement in *Pichia stipitis* fermentation of acid-hydrolyzed hemicellulose achieved by overliming (calcium hydroxide treatment) and strain adaptation. World. J. Microbiol. Biotechnol. 12(3):281-283.

Antonucci, S., M. Bravi, R. Bubbico, A.D. Michele and N. Verdone. 2001. Selectivity in citric acid production by *Yarrowia lipolytica*. Enzyme and Microbial Tech. 28(2/3):189-195.

Arzumanov, T.E., N.V. Shishkanova and T.V. Finogenova. 2000. Biosynthesis of citric acid by *Yarrowia lipoltyica* repeat-batch culture on ethanol. Appl. Microbiol. Biotechnol. 53 (5):525-529.

Balletsteros, J., J.M. Oliva, M. Ballesteros and J. Carrasco. 1993. Optimization of the simultaneous saccharification and fermentation process using thermotolerant yeasts. Appl. Biochem. Biotech. 39/40:201-211.

Banat, I.M. and R. Marchant. 1995. Characterization and potential industrial applications of five novel, thermotolerant, fermentative, yeast strains. World J. Microb. Biotech. 11:304-306.

Barnett, J.A., R.W. Payne and D. Yarrow. 2000. Yeasts: characteristics and identification. University Press, Cambridge, U.K.

Barth, G. and C. Gaillardin. 1996. *Yarrowia lipolytica*. In: *Nonconventional Yeasts in Biotechnology* (K. Wolf, ed). Springer-Verlag Berlin Heidelberg, New York, pp. 313-388.

Beckerich, J.M., P. Fournier, C. Gaillardin, H. Heslot, M. Rochet and B. Treton. 1984. Yeasts. In: *Genetics and breeding of industrial microorganisms* (C. Ball, ed). CRC Press Inc., Boca Raton, Florida, USA.

Belem, M.A.F., B.F. Gibbs and B.H. Lee. 1997. Enzymatic production of ribonucleotides from autolysates of *Kluyveromyces marxianus* grown on whey. J. Food Sci. 62(4):851-854.

Belem, M.A.F. and B.H. Lee. 1998. Production of bioingredients from *Kluyveromyces marxianus* grown on whey: an alternative. Critical Rev. Food Sci. Nutr. 38(7):565-598.

Benitez, T., J.M. Gasent-Ramirez, F. Castrejon and A.C. Codon. 1996. Development of new strains of yeast for the food industry. Biotechnol. Prog. 12:149-163.

Bhosale, P.B. and R.V. Gadre. 2001. Production of ß-carotene by a mutant of *Rhodotorula glutinis*. Appl. Microbiol. Biotechnol. 55:423-427.

Blandin, G, O. Ozier-Kalogeropoulos, P. Winker, F. Artinguenave and B. Dujon. 2000a. Genomic exploration of the hemiascomycetous yeasts: 16. *Candida tropicalis*. FEBS Letters 487(1):91-94.

Blandin, G., B. Llorente, A. Malpertuy, P. Wincker, F. Artinguenave and B. Dujon. 2000b. Genomic exploration of the hemiascomycetous yeasts: 13. *Pichia angusta* (FEBS 24377) FEBS Letters 487(1):76-81.

Bolotin-Fukuhara, M., C. Toffano-Nioche, F. Artinguenave, G. Duchateau-Nguyen, M. Lemaire, R. Marmeisse, R. Montrocher, C. Robert, M. Termier, P. Wincker and M. Wesolowski-Louvel. 2000. Genomic exploration of the hemiascomycetous yeasts: 11. *Kluyveromyces lactis* (FEBS 24375). FEBS Letters 487(1):66-70.

Boretsky, Y., A. Voronovsky, O. Liuta-Tehlivets, M. Hasslacher, S.P. Kohlwein and G.M. Shavlovsky. 1999. Identification of an ARS element and development of a high efficiency transformation system for *Pichia guilliermondii*. Curr. Genet. 36(4):215-221.

Buzzini, P. 2000. An optimization study of carotenoid production by *Rhodotorula glutinis* DBVPG 3853 from substrates containing concentrated rectified grape must as the sole carbohydrate source. J. Industrial Microb. Biotech. 24:41-45.

Buzzini, P. 2001. Batch and fed-batch carotenoid production by *Rhodotorula glutinis-Debaromyces castelli* co-cultures in corn syrup. J. Appl. Microb. 90:843-847.

Casaregola, S., C. Neuveglise, A. Leplinge, E. Bon, C. Feynerol, F. Artiguenave, P. Wincker and C. Gaillardin. 2000. Genomic exploration of the hemiascomycetous yeasts: 17. *Yarrowia lipolytica*. FEBS Letters 487(1):95-100.

Castoria, R., F. De Curtis, G. Lima and V. De Cicco. 1997. Beta-1, 3-glucanase activity of two saprophytic yeasts and possible mode of action as biocontrol agents against postharvest diseases. Postharvest Biol. Tech. 12(3):293-300.

Chan, H.Y. and K.P. Hon. 1999.Growth and carotenoid production by pH-stat cultures of *Phaffia rhodozyma*. Biotech. Letters 21(111):953-958.

Chand-Goyal, T. and R.A. Spotts. 1996. Control of postharvest pear diseases using natural saprophytic yeast colonists and their combination with a low dosage of thiabendazole. Postharvest Biol. Tech. 7(1/2):51-64.

Chang, C-C., C-S. Park and D.D.Y. Ryu. 1998. Improvement of heterologous protein productivity through a selected bioprocess strategy and medium design-a case study for recombinant *Yarrowia lipolytica* fermentation. Appl. Biochem. Biotech. 74(3):173-189.

Chaudhari, A.B. and S.B. Chincholkar. 1999. New osmotolerant *Schizosaccharomyces* for ethanol production. J. Food Sci. Techn., India 36(2):166-169.

Choi, J-H., K-H. Moon, Y-W. Ryu and J-H. Seo. 2000. Production of xylitol in cell recycle fermentations of *Candida tropicalis*. Biotech. Letters 22:1625-1628.

Chung, J.D. 2000. Design of metabolic feed controllers: application to high-density fermentations of *Pichia pastoris*. Biotech. Bioeng. 68(3):298-307.

Cruz, J.M. and J.C. Parajo.1998. Improved astaxanthin production by *Xanthophyllomyces dendrorhous* growing on enzymatic wood hydrolysates containing glucose and cellobiose. Food Chem. 63(4):479-484.

Daniel, H-J, R.T.Otto, M. Reuss and C. Syldatk. 1998. Sophorolipid production with high yields on whey concentrate and rapeseed oil without consumption of lactose. Biotech. Letters 20(8):805-807.

Daniel, H-J., R.T. Otto, M. Binder, M. Reuss and C. Syldatk. 1999. Production of sphorolipids from whey: development of a two-stage process with *Cryptococcus curvatus* ATCC 20509 and *Candida bombicola* ATCC 22214 using deproteinized whey concentrates as substrates. Appl. Microbiol. Biotechnol. 51:40-45.

D'Anjou, M and A.J. Daugulis. 2000. Mixed-feed exponent ional feeding for fed-batch culture of recombinant methylotrophic yeast. Biotech. Letters 22:341-346.

D'Anjou, M and A.J. Dauglis. 2001. A rational approach to improving productivity in recombinant *Pichia pastoris*. Biotech. Bioeng. 72(1):1-11.

De Baets, S. and E.J. Vandamme. 1999. Yeasts as producers of polysaccharides with novel application potential. SIM News 49(6):321-328.

De Felice, B., G. Pontecorvo and M. Carfagna. 1997. Degradation of waste waters from olive oil mills by *Yarrowia lipolytica* ATCC 20255 and *Pseudomonas putida*. Acta Biotechnol. 17(3):231-239.

Delgenes, J.P., M.C. Escare, J.M. Laplace, R. Moletta and J.M. Navarro. 1998. Biological production of industrial chemicals, i.e. xylitol and ethanol from lignocelluloses by controlled mixed culture systems. Industrial Crops and Products. 7(2/3):101-111.

Demain, A.L., H.J. Phaff and C.P. Kurtzman. 1998. The industrial and agricultural significance of yeasts. In: *The Yeasts, A Taxonomic Study* (C.P. Kurtzman and J. W. Fell, eds). 4th edition, Elsevier, Amsterdam, The Netherlands, pp. 13-19.

De Montigy, J., M-L. Straub, S. Potier, F. Tekaia, B. Dujon, P. Wincker, F. Artiguenave and J-L. Souciet. 2000. Genomic exploration of the hemiascomycetous yeasts: 8. *Zygosaccharomyces rouxii*. FEBS Lett. 487(1):52-55.

Deshpande, M. and L. Daniels. 1995. Evaluation of sophorolipid biosurfactant production by *Candida bombicola* using animal fat. Bioresource Technology 54:143-150.

De Winde, J.H. (ed.). 2003. *Topics in Current Genetics: Functional Genetics of Industrial Yeasts*. Springer-Verlag, Berlin, Germany, pp. 1-367.

Dhamija, S.S., D. Bhaskar and R. Gera. 1996. Thermotolerance and ethanol production (from 15% sucrose at 37°C) are at variance in mutants of *Schizosaccharomyces pombe*. Biotech. Letters 18(11):1341-1344.

Dujon, B. 2003. Comparative genomics of Hemiascomycetous yeasts: the systematic sequencing of *Candida glabrata*, *Kluyveromyces lactis*, *Debaromyces hansenii* and *Yarrowia lipolytica*. Plenary lecture 2-5 abstract.

Fabritius, D., H.J. Schaefer and A. Steinbuechel. 1996. Identification and production of cis-3-hydroxy-1, 18-octadec-9-enedioic acid by mutants of *Candida tropicalis*. Appl. Microbiol. Biotechnol. 45(3):342-348.

Ferrari, M.D., L. Loperena and H. Varela. 1996. Ethanol production from whey permeate using fed-batch culture of *Kluyveromyces fragilis*. Biotech. Letters 16(2):205-210.

Fleet, G. 2000. The biodiversity of yeasts in the production of foods and beverages. Plenary presentation abstract, ISY 2000, Arnhem, The Netherlands.

Florencio, J.A., C.R. Soccol, L.F. Furlanetto and T. Bonfim.1998. A factorial approach for a sugarcane juice-based low cost culture medium: increasing the astaxanthin production by the red yeast *Phaffia rhodozyma*. Bioprocess Eng.19(3):161-164.

Fontana, J.D., M.B. Chocial, M. Baron, M.F. Guimaraes, M. Maraschin, C. Ulhoa, J.A. Florencio and T.B. Bonfim. 1997. Astaxanthinogenesis in the yeast *Phaffia rhodozyma*-optimization of low-cost culture media and yeast cell-wall lysis. Appl. Biochem. Biotech. 63-65:305-314.

Freer, S.N., C.D. Skory and R.J. Bothast. 1998. Production of fuel ethanol from cellulosic biomass. Recent Res. Dev. Microbiol. 2(1):201-210.

Fukuda, H., T.Fujii and T. Ogawa. 1984. Microbial production of C-2 hydrocarbons, ethane, ethylene and acetylene. Agric. Biol. Chem. 48(5):1363-1365.

Gabrinska-Loniewska, A., E. Pajor and E. Slavikova. 1997. Comparative analysis of yeast-like species potentially useful to environmental biotechnology. 1997. Acta Mycologica 32(1):99-106.

Gellissen, G. 2000. Heterologous protein production in methylotrophic yeasts. Appl. Microb. Biotech. 54(6):741-750.

Giga-Hama, Y. 1997. Fission yeast *Schizosaccharomyces pombe*: an attractive host for heterologous protein production. In: *Foreign Gene Expression in Fission Yeast: Schizosaccharomyces pombe* (Y. Giga-Hama and H. Kumagai, eds). Springer-Verlag, Germany, pp. 3-28.

Giuseppe, L., D.C. Filippo, C. Raffaello and D.C. Vincenzo. 1998. Activity of the yeasts *Cryptococcus laurentii* and *Rhodotorula glutinis* against post-harvest rots on different fruits. Biocontrol Sci. Tech. 8(2):257-267.

Grbin, P.R. and P.A. Henschke. 2000. Mousy off-flavour production in grape juice and wine by *Dekkera* and *Brettanomyces* yeasts. Aust. J. Grape Wine Res. 6(3):255-262.

Hansen, H. and C.P. Hollenberg. 1996. *Hansenula polymorpha* (*Pichia angusta*). In: *Nonconventional Yeasts in Biotechnology* (K. Wolf, ed). Springer-Verlag, Berlin, Germany, pp. 293-311.

Hassan, M.A. and S.H. El-Sharkway. 1999. Microbial production of useful fatty acids from used sunflower oil. Zagazig J. Pharm. Sci. 8(2):46-49.

Hayashida, Y., K. Nishimura and J.C. Slaughter. 1998.The importance of the furanones HDMK and HEMF in the flavour profile of Japanese barley miso and their production during fermentation. J. Food Sci. Agric. 78(1):88-94.

Hollenberg, C.P., and G. Gellissen.1997. Production of recombinant proteins by methylotrophic yeasts. Current Opinions in Biotechnology 8:554-560.

Huang, C. Jr., S.L. Lee and C.C. Chou. 2000. Production and molar yield of 2-phenylethanol by *Pichia fermentans* L-5 as affected by some medium components. J. Biosc. Bioeng. 90(2):142-147.

Kesava, S.S., G-H. An, C-H. Kim, S-K. Rhee and E-S. Choi. 1998. An industrial medium for improved production of carotenoids from a mutant strain of *Phaffia rhodozyma*. Bioprocess Eng. 19(3):165-170.

Kim, J-H., Y-W. Ryu and J-H. Seo. 1999. Analysis and optimization of a two-substrate fermentation for xylitol production using *Candida tropicalis*. J. Indust. Microb. Biotech. 22(3):181-186.

Kruse, B. and K. Schuegerl. 1996. Investigation of ethanol formation by *Pachysolen tannophilus* from xylose and glucose/xylose co-substrates. Process Biochem. 31(4):389-407.

Kunze, G. and I. Kunze. 1996. *Arxula adeninivorans*. In: *Nonconventional Yeasts in Biotechnology* (K. Wolf, ed). Springer, Berlin, Germany, pp. 389-409.

Kurtzman, C.P. and J.W. Fell. 1998. *The Yeasts, A Taxonomic Study* (C.P. Kurtzman and J. W. Fell, eds), 4th edition. Elsevier, Amsterdam, The Netherlands.

Kurtzman, C. 2001. Six new anamorphic ascomycetous yeasts near *Candida tanzawaensis*. FEMS Yeast Research 1:177-185.

Kurtzman, C. and J. Sugiyama. 2001. Ascomycetous yeasts and yeastlike taxa. In: *The Mycota VII Part A. Systematics and Evolution* (McLaughlin, McLaughlin and Lemke, eds.) Springer-Verlag, Berlin, Germany, pp. 179-200.

Kyong, S.H. and C.S. Shin. 2000. Optimized production of L-ß-hydroxybutyric acid by a mutant of *Yarrowia lipolytica*. Biotechnol. Lett. 22(13):1105-1110.

Labrou, N. and D.J. Rigden. 2001. Active-site characterization of *Candida boidinii* formate dehydrogenase. Biochem. J. 354(2):455-463.

Lachance, M-A. and W.T. Starmer. 1998. Ecology and yeasts. In: *The Yeasts, A Taxonomic Study* (C.P. Kurtzman and J. W. Fell, eds), 4th edition. Elsevier, Amsterdam, The Netherlands, pp. 21-30.

Laires, A., I. Spencer-Martins and N. van Uden. 1983. Use of D-glucosamine and 2-deoxyglucose in the selective isolation of mutants of the yeast *Lipomyces starkeyi* derepressed for the production of extracellular endodextranase. Zeitschrift fur Allgemeine Mikrobiologie 23(9):601-603.

Lang, S., A. Brakemeier, U. Rau and F. Wagner. 1996. Biotechnological production of interfacial active glycolipids. Oils-Fats-Lipids 1995, Proc. World. Congr. Int. Soc. Fat. (21st) 1:53-54.

Lark, N., Y. Xia, C-G. Qin, C.S. Gong and G.T. Tsao. 1997. Production of ethanol from recycled paper sludge using cellulase and yeast, *Kluyveromyces marxianus*. Biomass and Bioenergy 12(2):135-143.

Lepingle, A., S. Casaregola, C. Neuveglise, E. Bon, H-V. Nguyen, F. Artiguenave, P. Wincker and C. Gaillardin. 2000.Genomic exploration of the hemiascomycetous yeasts: 14. *Debaromyces hansenii* var *hansenii*. FEBS Letters 487(1):82-86.

Liiv, L., P. Parn and T. Alamae. 2001. Cloning of the maltase gene from methylotrophic yeast, *Hansenula polymorpha*. Gene 265(1/2):77-85.

Lim, S.H., J.S. Choi and Y.E. Park. 2001. Microbial production of riboflavin using riboflavin overproducers, *Ashbya gossypii*, *Bacillus subtilis* and *Candida famata*. An overview. Biotechnol. Bioprocess. Eng. 6(2):75-88.

Lima, G., F. De Curtis, R. Castoria and V. De Cicco. 1998. Activity of the yeasts *Cryptococcus laurentii* and *Rhodotorula glutinis* against post-harvest rots in different fruits. Biocontrol Sci. Tech. 8(2):257-267.

Limtong, S., S. Deejing and W. Santisoparsi. 1998. Construction of high ethanol halotolerant hybrid by intergeneric protoplast fusion of *Saccharomyces cerevisiae* and *Zygosaccharomyces rouxii*. Kaseterat J. Nat. Sci. 32(2):213-223.

Linardi, V.R., J.C.T. Dias and C.A. Rosa. 1996. Utilization of acetonitrile and other aliphatic nitriles by a *Candida famata* strain. FEMS Microbiol. Lett. 144(1):67-71.

Llorente, B., A. Malpertuy, G. Blandin, F. Artinguenave, P. Wincker and B. Dujon. 2000. Genomic exploration of the hemiascomycetous yeasts: 12. *Kluyveromyces marxianus var. marxianus* (FEBS 24376) FEBS Letters 487(1):71-75.

Martinez, X.C., A. Narbad, A.T. Carter and M. Stratford. 1996. Flocculation of the yeast *Candida famat*a (*Debaromyces hansenii*): an essential role for peptone. Yeast 2(5):415-423.

Matelli, H.L., I.M. da Silva, N.O. Souza and D. Pomeroy. 1990. Production of ß-carotene by a *Rhodotorula* strain grown on sugar cane juice. Biotechnology Letters 12(3):207-208.

Matsuyama, A., H. Yamamoto, N. Kawada and Y. Kobayashi. 2001. Industrial production of R-1,3-butanediol by new catalysts. J. Mol. Catal. B: Enzym. 11:4-6.

Mauersberger, S., M. Ohkuma, W-H. Schunck and M. Takagi. 1996. *Candida maltosa*. In: *Nonconventional Yeasts Biotechnol* (K. Wolf, ed.). Springer, Berlin, Germany, pp. 411-580.

Mayer, A.F., K. Helmuth, H. Schlieker, R. Lopez-Ulibarri, S. Oertel, U. Dahlems, A.W.M. Strasser and A.P.G.M. van Loon. 1999. An expression system matures: a highly efficient and cost-effective process for phytase production by recombinant strains of *Hansenula polymorpha*. Biotech. Bioeng. 63(3):373-381.

Minning, S., A. Serrano, P. Ferrer, C. Sola, R.D. Schmid and F. Valero. 2001. Optimization of the high-level production of *Rhizopus oryzae* lipase in *Pichia pastoris*. J. Biochem. 86(1):59-70.

Miura, Y., K. Kondo, T. Saito, H. Shimada, P. Fraser and N. Misawa. 1998. Production of the carotenoids lycopene, ß-carotene and astaxanthin in the food yeast *Candida utilis*. Appl. Envt. Microbiol. 64(4):1226-1229.

Miura, Y., K. Kondo, H. Shimada, T. Saito, K. Nakamura and N, Misawa. 1998. Production of lycopene by the food yeast, *Candida utilis* that does not naturally synthesize carotenoid. Biotech. Bioeng. 58(2/3):306-308.

Miura, Y., M. Kettoku, M. Kato, K. Kobayashi and K. Kondo. 1999. High level production of thermostable α-amylase from *Sulfolobus solfataricus* in high-cell density culture of the food yeast *Candida utilis*. J. Mol. Microbiol. Biotechnol. 1(1):129-134.

Montesinos, J.L., E. Dalamu and C. Casas. 2003. Lipase production in continuous culture of

Candida rugosa. J. Chem. Tech. Biotech. 78:753-761.

Nadezda, K., M. Maria and C. Peter. 1997. Structure of glucomannan-protein complex from the yeast *Cryptococcus laurentii*. J. Carboh. Chem. 16(5-4):609-623.

Nakamura, T., T. Suzuki, J. Junko, N. Kato, Y Sakai, D. Mochizuki and H. Takahashi. 2000. Production of recombinant phytase by fed-batch fermentation of *Candida boidinii*. US Patent 6,140,077.

Nakano, K., R. Katsu, K. Tada and M. Matsumura. 2000. Production of highly concentrated xylitol by *Candida magnoliae* under a microaerobic condition maintained by a simple fuzzy control. J. Bios. Bioeng. 89(4):372-376.

Nielsen, J. and L. Olsson. 2002. An expanded role for microbial physiology in metabolic engineering and functional genomics: moving towards systems biology. FEMS Yeast Research 2:175-181.

Nishikawa, A., T. Sugita and T. Shinoda. 1997. Differentiation between *Debaromyces hansenii/Candida famat*a complex and *Candida guilliermondii* by polymerase chain reaction. FEMS Immunol. Med. Microbiol. 19(2):125-129.

Nishikawa, A., T. Sugita and T. Shinoda. 1999. Rapid identification of *Debaromyces hansenii/ Candida famata* by polymerase chain reaction. Med. Mycol. 37(2):101-104.

Oh, D-K. and S-Y. Kim. 1998. Increased xylitol yield by feeding xylose and glucose in *Candida tropicalis*. Appl. Microbiol. Biotech. 50(4):419-425.

Panchall, C.J., I. Russell, A.M. Sills and G.G. Stewart. 1984. Genetic manipulation of brewing and related yeast strains. Food Tech., February, pp. 99-111.

Parajo, J.C., V. Santos and M. Vasquez. 1998a. Optimization of carotenoid production by *Phaffia rhodozyma* cells grown on xylose. Process Biochem. 33(2):181-187.

Parajo, J.C., V. Santos and M. Vazquez. 1998b. Production of carotenoids of *Phaffia rhodozyma* growing on media made from hemicellulosic hydrolyzates of *Eucalyptus globules* wood. Biotechnol. Bioeng. 59 (4):501-506.

Parrondo, J., L.A. Garcia and M. Diaz. 2000. Production of an alcoholic beverage by fermentation of whey permeate with *Kluyveromyces fragilis*. I. Primary metabolism. J. Inst. Brewing 106(6):367-375.

Pazouki, M., P.A. Felse, J. Sinha and T. Panda. 2000. Comparative studies on citric acid production by *Aspergillus niger* and *Candida lipolytica* using molasses and glucose. Bioprocess Eng. 22(4):353-361.

Penttila, M. 1997. In: *Biotechnology in the food chain*. VTT Symposium 177. pp. 109-118.

Phaff, H.J. 1985. Biology of yeasts other than *Saccharomyces*. Biotech. Series 6:537-562.

Picataggio, S., T. Rorer, K. Deanda, D. Lawning, R. Reynolds, J. Mielenz and L.D. Dudley. 1992. Metabolic engineering of *Candida tropicalis* for the production of long-chain fatty acids. Biotech. 10:894-898.

Possoth, V., M. Zimmermann and U. Klinner. 1996. Peculiarities of the regulation of fermentation and respiration in the Crabtree-negative, xylose-fermenting yeast *Pichia stipitis*. Appl. Biochem. Biotechnol. 57/58:201-212.

Pribylova, L. and H. Sychrova. 2003. Osmotic stress resistant yeast *Zygosaccharomyces rouxii*-isolation of auxotrophic mutants and efficient transformation by electroporation. Abstract 11-7. XXIst International Conference on Yeast Genetics and Molecular Biology, Gothenburg, Sweden.Yeast 20(S1).

Ramirez, J., M.L.Nunez and R. Valdivia. 2000. Increased astaxanthin production by a *Phaffia rhodozyma* mutant grown on date juice from *Yucca fillifera*. J. Indust. Microb. Biotech. 24(3):187-190.

Ramos, J. V. Montiel, C. Prista, R. Garcia-Salcedo and M.C. Loureiro-Dias. 2003. Abstract 11-46. Yeast 20(S1), XXI International Conference on Yeast Genetics and Molecular Biology, Gothenburg, Sweden.

Rau, U., R. Heckmann, V. Wray and S. Lang. 1999. Enzymatic conversion of a sophorolipid into a glucose lipid. Biotech. Letters 21:973-977.

Rau, U., S. Hammen, R. Heckmann, V. Wray and S. Lang. 2001. Sophorolipids: a source for novel compounds. Industrial Crops and Products 13:85-92.

Rodriguez, E., K. Sanchez, H. Roca, B. Garcia, J. Cremata and J. Delgado. 1996. Procedure for the high-level recombinant dextranase production. Biotecnol. Apl. 13(3):201.

Rodrigues, D.C.G.A., S.S. Silva, A.M.R. Prata and M.G.A. Felipe. 1998. Biotechnological production of xylitol from agroindustrial residues. Evaluation of bioprocesses. Appl. Biochem. Biotech. 70-72:869-875.

Roostita, R. and G.H. Fleet. 1999. Growth of yeasts isolated from cheese on organic acids in the presence of sodium chloride. Food Technol. Biotechnol. 37(2):73-79.

Saha, B.C. and R.J. Bothast. 1996. Production of L-arabitol from L-arabinose by *Candida entomaea* and *Pichia guilliermondii*. Appl. Microbiol. Biotechnol 45(3):299-306.

Sakai, Y, M. Akiyama, H. Kondoh, Y. Shibano and N. Kato. 1996. High-level secretion of fungal glucoamylase using the *Candida boidinii* gene expression system. Biochimica et Biophysica Acta 1308(1):81-87.

Sakai, Y., Y. Tani and N. Kato. 1998. Biotechnological application of cellular functions of the methylotrophic yeast. J. Molecular Catal. B Enzymatic 6(3):161-173.

Sanchez, S., V. Bravo, E. Castro, A.J. Moya and F. Camacho. 1998. The production of xylitol from D-xylose by fermentation with *Hansenula polymorpha*. Appl. Microb. and Biotech. 50(5):608-611.

Sanchez, S., V.Bravo, E. Castro, A.J. Moya and F. Camacho. 1999. Comparative study of the fermentation of D-glucose/D-xylose mixtures with *Pachysolen tannophilus* and *Candida shehatae*. Bioprocess Eng. 21(6):525-532.

Sasahara, H., M. Mine and K. Izumori. 1998. Production of D-talitol from D-psicose by *Candida famata* R28. J. Ferment. Bioeng. 85(1):84-88.

Santopietro, L.M.D., J.F.T. Spencer and F. Sineriz. 1998. Fed-batch and continuous culture of *Phaffia rhodozyma* (*Xanthophyllomyces dendrorhous*). Folia Microbiol. 43(2):169-172.

Sberna, G., R. Cappai, A. Henry and D.H. Small. 1996. Advantages of the methylotrophic yeast *Pichia pastoris* for high-level expression and purification of heterologous proteins. Australas. Biotechnol. 6(2):82-87.

Schwan, R.F., R.M. Cooper and A.E. Wheals. 1997. Endopolygalacturonase secretion by *Kluyveromyces marxianus* and other cocoa pulp-degrading yeasts. Enzyme and Microbial Tech. 21:234-244.

Sharyshev, A.A., E.V. Peskova and G.N. Komarova. 1997.Effect of cultivation conditions on the level of enzymes of n-alkane metabolism in *Candida maltosa* cells. Microbiology (Moscow) 66(6):652-656.

Shimada, H., K. Kondo, P. Fraser, Y. Miura, T. Saito and N. Misawa. 1998. Increased carotenoid production by the food yeast *Candida utilis* through metabolic engineering of the isoprenoid pathway. Appl. Envt. Microbiol. 64(7):2676-2680.

Sibirny, A.A. 1996a. *Pichia guilliermondii*. In: *Nonconventional Yeasts in Biotechnology* (K. Wolf, ed). Springer-Verlag, Berlin, Heidelberg, New York, pp. 255-275.

Sibirny, A. 1996. *Pichia methanolica* (*Pichia pinus* MH4). In: *Nonconventional yeasts in biotechnology* (K. Wolf, ed.) Springer-Verlag, Berlin, Germany, pp. 277-291.

Silva, S.S., I.C. Roberto, M.G.A. Felipe and I.M. Mancilha. 1996. Batch fermentation of xylose for xylitol production in a stirred tank bioreactor. Process Biochem. 31(6):549-553.

Singh, D., P. Nigam, I.M. Banat, R. Marchant and A.P. McHale. 1998. Review: ethanol production at elevated temperatures and alcohol concentrations: Part II-use of *Kluyveromyces marxianus* IMB3.World J. Microb. Biotech. 14(6):823-834.

Skory, C.D., S.N. Freer and R.J. Bothast. 1996. Properties of an intracellular beta-glucosidase purified from the cellobiose-fermenting yeast *Candida wickerhamii*. Appl. Microbiol. Biotechnol. 46(4):353-359.

Sreekrishna, K. and K.E. Kropp. 1996. *Pichia pastoris*. In: *Nonconventional Yeasts in Biotechnology* (K.Wolf, ed). Springer-Verlag, Berlin, Germany, pp. 203-253.

Sreenath, H.K. and T.W. Jeffries. 1999. Production of ethanol from wood hydrolyzate by yeasts. Bioresour. Technol. 72(3):253-260.

Stahmann, K-P, J.L. Revuelta and H. Seulberger. 2000. Three biotechnical processes using *Ashbya gossypii*, *Candida famat*a, or *Bacillus subtilis* compete with chemical riboflavin production. Appl. Microbiol. Biotechnol. 53(5):509-516.

Suryadi, H., T. Katsuragi, N. Yoshida, S. Suzuki and Y. Tani. 2000. Polyol production by culture of methanol-utilizing yeast. J. Biosc. Bioeng. 89(3):236-240.

Sugar, D. and R.A. Spotts. 1999. Control of postharvest decay in pear by four laboratory-grown yeasts and two registered biocontrol products. Plant Disease 83(2):155-158.

Sung-Wook, Y., J-B. Park, N.S. Han, Y-W. Ryu and J-H. Seo. 1999. Production of erythritol from glucose by an osmophilic mutant of *Candida magnoliae*. Biotech. Letters 21(10):887-890.

Sychrova, H., V. Braun, S. Potier and J-L. Souciet. 2000. Organization of specific genomic regions of *Zygosaccharomyces rouxii* and *Pichia sorbitophila*: comparison with *Saccharomyces cerevisiae*. Yeast 16(15):1377-1385.

Tanaka, A., R. Yamada, S. Shimizu and S. Fukui. 1971. Studies on the formation of vitamins and their functions in hydrocarbon fermentations. J. Ferment. Technol. 49(9):792-802.

Thorpe, E.D., M. d'Anjou and A.J. Daugulis. 1999. Sorbitol as a non-repressing carbon source for fed-batch fermentation of recombinant *Pichia pastoris*. Biotech. Letters 21:669-672.

Trujillo, L.E., J.G. Arrieta, F. Dafhnis, J. Garcia, J. Valdes, Y. Tambara, M. Perez and L. Hernandez. 2001. Fructo-oligosaccharides production by the *Gluconacetobacter diazotrophicus levansucrase* expressed in the methylotrophic yeast *Pichia pastoris*. Enzyme and Microbial Tech. 28(2/3):139-144.

Vandeska, E., S. Amartey, S. Kuzmanova and T.W. Jeffries. 1996. Fed-batch culture for xylitol production by *Candida boidinii*. Process Biochem. 31(3):265-270.

Vazquez, M., V. Santos and J.C.Parajo.1998. Fed-batch cultures of *Phaffia rhodozyma* in xylose-containing media made from wood hydrolyzates. Food Biotechnol. 12(1/2):43-55.

Vijayakumar, A.R., E. Csoeregi, A. Heller and L. Gordon. 1996. Alcohol biosensors based on coupled oxidase-peroxidase systems. Anal. Chim. Acta 327(3):223-234.

Visser, H., J. Verdoes, G. Sandmann and J. van den Berg. 2003. Genetical engineering of the carotenoid biosynthetic pathway in *Xanthophyllomyces dendrorhous*. Abstract 12-46.Yeast 20(S1). XXI International Conference on Yeast Genetics and Molecular Biology, Gothenburg, Sweden.

Voronovsky, A., C.A. Abbas, L.R.Fayura, B.V. Kshanovska, K.V. Dmytruk, K.A. Sybirna and A.A. Sibirny. 2002. Development of a transformation system for the flavinogenic yeast *Candida famata* FEMS. Yeast Res. 2(3):381-388.

Wache, Y., M. Aguedo, J-M. Nicaud and J-M. Belin. 2003. Catabolism of hydroxyacids and biotechnological production of lactones by *Yarrowia lipolytica*. Appl. Microbiol. Biotech. 61:393-404.

Wartmann, T. and G. Kunze. 2000. Genetic transformation and biotechnological application of the yeast *Arxula adeninvorans*. Appl. Microb. Biotech. 54(5):619-624.

Wery, J., J.C. Verdoes and A.J.J. Van Ooyen. 1998. Efficient transformation of the astaxanthin-producing yeast *Phaffia rhodozyma*. Biotechno. Tech. 12(5):399-405.

Wesolowski-Louvel, M., K. Breunig and H. Fukuhara. 1996. *Kluyveromyces lactis*. In: *Nonconventional Yeasts in Biotechnology* (K. Wolf, ed). Springer-Verlag, Berlin, Heidelberg, New York, pp. 139-201.

Wolf, K. 1996. In: *Nonconventional Yeasts in Biotechnology. A Handbook*. Springer-Verlag, Berlin, Heidelberg, New York.

Wolf, K., K. Breunig and G. Barth (eds). 2003. *Non-conventional Yeasts in Genetics, Biochemistry and Biotechnology*. Springer-Verlag, Berlin, Germany. pp. 1-494.

XXI International Conference on Yeast Genetics and Molecular Biology, July 7-12, Gothenburg, Sweden. Published in Yeast 20(S1) (J.M. Cherry and S. Hohmann, eds).

Yahashi, Y., H. Horitsu, K. Kawai, T. Suzuki and K. Takamizawa. 1996. Production of xylitol from D-xylose by *Candida tropicalis*: the effect of D-glucose feeding. J. Ferment. Bioeng. 81(2):148-152.

Yamada, R-H, Y. Kera, H. Toi, T. Hayashi, K. Arimoto, M. Takahashi, I. Iwazaki and S. Yamashita. 2001. Microbial oxidases of acidic D-amino acids. J. Molecular Catalysis B: Enzymatic 12(1-6):93-104.

Yamazaki,T., A. Kubiki, H. Ota, T.M. Mitzel, L.A. Paquette and T. Sugai. 2000. Yamadazyma farinosa IFO 10896-mediated reduction of 4,4-dimethoxy-2-butanone as the key step for the preparation of 1,3- diols with unsymmetrical substituents. Synth. Commun. 30(16):3061-3072.

Yanai, T. and M. Sato. 2000. Purification and characterization of an α-L-rhamnosidase from *Pichia angusta* X349. Bioscience, Biotech. Biochem. 64(10):2179-2185.

Yurimoto, H., T. Komeda, C.R. Lim, T. Nakagawa, K. Kondo, N. Kato and Y. Sakai. 2000. Regulation and evaluation of five methanol-inducible promoters in the methylotrophic yeast *Candida boidinii*. Biochimica et Biophysica Acta 1493(1-2):56-63.

Beverage alcohol production

Chapter 14

Production of Scotch and Irish whiskies: their history and evolution

T. PEARSE LYONS

President, Alltech Inc., Nicholasville, Kentucky, USA

Introduction

Whisky is the potable spirit obtained by distillation of an aqueous extract of an infusion of malted barley and other cereals that has been fermented with strains of *Saccharomyces cerevisiae* yeast. Various types of whisky are produced in a number of different countries in the world. They differ principally in the nature and proportion of the cereals used as raw materials along with malted barley, and also in the type of still used for distillation.

The principal types of whisky are also characteristic of particular geographical regions of the world. In Scotland, the characteristic product is manufactured using only malted barley as the raw material; and the fermented malt mash is distilled in relatively small pot stills.

The product, known as Scotch malt whisky, is produced in small distilleries, of which there are over 100 in Scotland. Scotch malt whisky is marketed both as a straight malt whisky, many brands of which have recently become extremely popular throughout the world, and also as a blend with another type of whisky produced in Scotland known as 'Scotch grain whisky' or (because it is distilled continuously in Coffey-type patent stills) as 'patent-still whisky'. Most Scotch whiskies available on the international market consist of blends with 20-30% malt whisky and 70-80% grain whisky. Within the blend, there may be as many as 20-30 individual malt whiskies and grain whiskies. These blends are, by law, matured for at least three years but in practice this period is much longer. Unblended Scotch malt whiskies are usually matured for a minimum of eight years.

The cereals used in the manufacture of Scotch grain whisky are malted barley, together with a high proportion (up to 90%) of wheat or corn (maize). Currently wheat is the main cereal, chosen on the basis of cost and the attraction of using a Scottish-grown cereal. All whiskies are legally protected and defined, mainly because of the huge revenues that governments obtain from their sale. The Scotch Whisky Order (1990) and the Scotch Whisky Act (1988), in defining Scotch Whisky, state that to be called Scotch Whisky spirits must be:

1. produced at a distillery in Scotland
2. produced from water and malted barley to which only whole grains or other cereals may be added
3. processed at that distillery into a mash
4. converted to fermentable carbohydrate only by endogenous enzymes
5. fermented only by the addition of yeast

6. distilled to an alcohol strength less than 94.8% so that the distillate has the aroma and taste derived from the raw materials
7. matured in Scotland in oak casks of less than 700 litres for a minimum of three years
8. be a product that retains the color, aroma and taste derived from the raw materials
9. produced with no substance other than water and spirit caramel added

The word 'Scotch' in this definition is of geographical and not generic significance.

Irish whiskey (spelled with an e) is a distinctive product of either the Republic of Ireland or of Northern Ireland. In the Republic of Ireland, definitions were enacted by the Parliament in the Irish Whiskey Acts of 1950 and 1980. The 1950 Act distinguished pot still whiskey from blends and stated that the title 'Irish Pot-Still Whiskey' was reserved solely for spirits distilled in pot stills in the Republic from a mash of cereal grains normally grown in that country and saccharified by a diastase of malted barley. The 1980 legislation specified that the term 'Irish Whiskey' only applied to 'spirits distilled in the Republic or Northern Ireland from a mash of cereals saccharified by the diastase of malt contained therein, with or without other natural diastases'. This meant that unlike Scotch, Irish whiskey may be produced with the use of microbial enzyme preparations in addition to malt. The 1980 Act also specified that the whiskey must be aged for at least three years in Ireland in wooden barrels. While not possessing the 'smoky' taste and aroma of Scotch, Irish whiskey is usually more flavorful and has a heavier body than Scotch. Moreover, the whiskey is distilled not twice, as in Scotland, but three times to give a very strong spirit of 86° GL compared with the 71° GL whisky distilled in Scotland.

The tremendous popularity of whiskies manufactured in Scotland, Ireland, the US and Canada has prompted several other countries to try manufacturing whiskies, usually ones designed to resemble Scotch. Indeed, the number of countries with minor but nevertheless significant whisky-distilling industries must now be well over a dozen. Some countries, notably Australia and Japan, have whisky-distilling industries producing a sufficiently acceptable product for them to venture into the export industry. Other countries, including the Netherlands and Spain, have whisky-distilling industries that cater mainly, if not exclusively, for home consumption. The measures that some of these industries have taken to imitate Scotch become apparent when the spirits are sampled. One of the two whisky distilleries in Spain located northwest of Madrid in the Guadarrama Hills near Segovia produces a very acceptable Scotch-type whisky. Its quality is attributed in part to the fact that the water used, which comes from the surrounding hills, closely resembles that used in highland Scotch whisky distilleries.

Recommended descriptions of Scotch whisky manufacture have come from Brander (1975), Daiches (1969), Dunnett (1953), Gunn (1935), Robb (1950), Ross (1970), Simpson (1968), Simpson *et al.* (1974), Ross Wilson (1959, 1970 and 1973) and Philip (1989). There are a few smaller books describing individual distilleries in Scotland. Recommended are those by McDowell (1975) and Wilson (1973; 1975). Recommended reading on Irish whiskey includes an account by McGuire (1973) and short review articles by Court and Bowers (1970) and Lyons (1974).

History of whisky production

The origins of the art of distilling potable spirits are obscure, and probably date back to ancient China. However, the first treatise on distilling was written by the French chemist Arnold de Villeneuve around 1310. The potency of distilled spirits caused many to be known as the 'water of life', a description that survives today in such names as *eau de vie* for French brandy and *akvavit* and *aquavit* for spirits in Northern Europe. The name 'whisky' is a corruption of 'uisgebaugh', the Gaelic word for water of life. Uisge was corrupted first into 'usky', which finally became whisky after several centuries. Dr. Johnson sang the praises of this potable spirit, although in his Dictionary of 1755 it is listed under 'u' and not 'w'.

Much to the chagrin of the Scotsman, it is likely that the first whisky was distilled not in Scotland but in Ireland. The spirit was known in Ireland when that land was invaded by the

English in 1170. In all likelihood the art of distillation was imported into Scotland by missionary monks from Ireland. Two of today's main centers of Scotch distilling, namely the island of Islay and the Speyside town of Dufftown, were the sites of early monastic communities.

Whisky, principally Scotch whisky, has for many years been one of the most popular distilled beverages in the world; and it was in Scotland rather than in Ireland that its qualities came to be extensively appreciated. This has continued to the present day and in the intervening period many Scotsmen have felt compelled to record for posterity their thoughts and inspirations on the potable spirit. There are numerous histories of whisky distilling in Scotland, some more comprehensive than others. For good general accounts, the reader is referred to the texts by Brander (1975), Daiches (1969), Ross (1970) and Ross Wilson (1970).

Whisky distilling flourished in Scotland not least because consuming the spirit helped the inhabitants to withstand the climatic rigors of this northern region of Britain. The first recorded evidence of whisky production in Scotland is an entry in the Exchequeur Rolls for the year 1494. It reads "To Friar John Cor, by order of the King, to make aquavitae, eight bolls of malt". Production of whisky was therefore being controlled; and an Act of 1597 decreed that only earls, lords, barons and gentlemen could distill for their own use. To many Scots of this era whisky was a medicine, and in 1506 King James IV of Scotland had granted a monopoly for manufacture of 'aqua vitae' to the Guild of Barber Surgeons in the City of Edinburgh.

Taxation on whisky production first appeared in the 17th century. Breaches of the monopoly regulations and the need to raise money to send an army into England to help the English Parliament in its war against Charles I led to the Act of 1644, which fixed a duty of two shillings and eight pence Scot's on a pint of whisky (the Scot's pint was then about 1.5 litres). But the tax was short-lived and was replaced by a malt tax that later was also repealed.

At the time of the Treaty of Union between Scotland and England in 1707 there was a tax on malt in England, but not in Scotland. The English were irate; and in 1725 when Lord Walpole's administration decided to enforce the tax in Scotland, the first of a series of Malt Tax riots occurred. The English, meanwhile, had cultivated a taste for French brandy, there being very little whisky consumed at that time outside of Scotland. However around 1690 William III began to wage commercial war against the French, and imposed punitive taxes on imports of French brandy into England. The English reacted by acquiring a taste for gin, which was distilled locally. The scale of drunkenness that developed with the popularity of gin had to be controlled by law; and the Acts of 1736 and 1713 levied high taxes on gin manufacturers. Both of these acts contained clauses exempting Scotland, but not for long. The Parliament in London saw the prospect of a rich harvest of taxes in the distilleries of Scotland; and in a series of acts starting in 1751, production of whisky in Scotland was increasingly subjected to taxation.

The outcome of these punitive measures was not surprising. An extensive and thriving business in illicit distillation of whisky grew up in Scotland as described by Sillett (1965). Curiously, illicit production of Scotch hardly extended over the border into England, although there are a few records of the operation of illicit stills in the Cheviot Hills west of Newcastle-upon-Tyne. Following the Act of 1823, which introduced much stiffer penalties for illicit distillation, and to some extent because of the increased standards of living in northern Scotland, illicit manufacture of whisky declined. Indeed, many erstwhile illicit distillers emerged to become legal and registered distillers of Scotch whisky.

In 1826, Robert Stein of the Kilbagie distillery in Clackmannanshire, Scotland, patented a continuously operating still for whisky production. However, this invention was superseded in 1830 with the introduction by Aeneas Coffey of an improved version of this type of still. The appearance of continuous stills sparked off a period of turmoil in the Scotch whisky industry, it being claimed that the product from the continuous distillation of a mash that contained unmalted grain (described as neutral or 'silent spirit') could not be called whisky, since it had not been distilled in the traditional pot still. The battle was waged for about three quarters of a century; and in 1908 a Royal Commission decided that malt whisky and grain neutral spirit, when blended, could be labeled whisky.

The major factors which have affected the development of the whisky distilling industry in Scotland in this century have been economic. The industry has had to endure the privations of two world wars, the economic depression in Great Britain during the 1920s and prohibition in the United States from 1920 to 1933, which greatly affected export of Scotch to North America. Since 1945, however, the industry in Scotland has consolidated and expanded. The 20th century has also witnessed a considerable improvement in the quality of whisky distilled and blended in Scotland as a result of the acceptance of blending malt whiskies with grain whisky and the amalgamation of several smaller distilleries into combines.

Scotch malt whiskies can be divided into 'highland', 'lowland', 'Islay' and 'Campbeltown whiskies' (Simpson *et al.*, 1974). The 'highland line', which separates the areas in Scotland in which the first two types of spirit are distilled, is a straight line which runs from Dundee in the east to Greenock in the west (Figure 1). It then extends southwards, below the Isle of Arran. Any whisky produced north of this line, including those from Campbeltown and Islay, is entitled to be called a highland malt whisky, while whiskies distilled in areas south of the line are designated as lowland whiskies. Of the 104 malt whisky distilleries in Scotland, 95 are highland malt whisky distilleries. Of these, no fewer than 49 are situated in an area measuring 50 miles east to west and 20 miles southwards from the Moray Firth. This area of Speyside has been called the 'Kingdom of Malt Whisky' (Cameron Taylor, 1970). Classification of the four whiskies distilled on the islands of Jura, Orkney and Skye is disputed. Some authorities list them along with the Islay whiskies as 'island' whiskies, others as highland whiskies, which is geographically correct. There are also eight grain whisky distilleries in Scotland.

Whiskey distilling in Ireland was, as has been noted, first recorded in the 12th century. By 1556, it had become sufficiently widespread to warrant legislation to control it. A statute proclaimed that year stated that a license was required to manufacture the spirit, but that peers, gentlemen owning property worth £10 or more and borough freemen were exempt (McGuire, 1973). Taxation of whisky distilling gradually became more excessive and collection of taxes

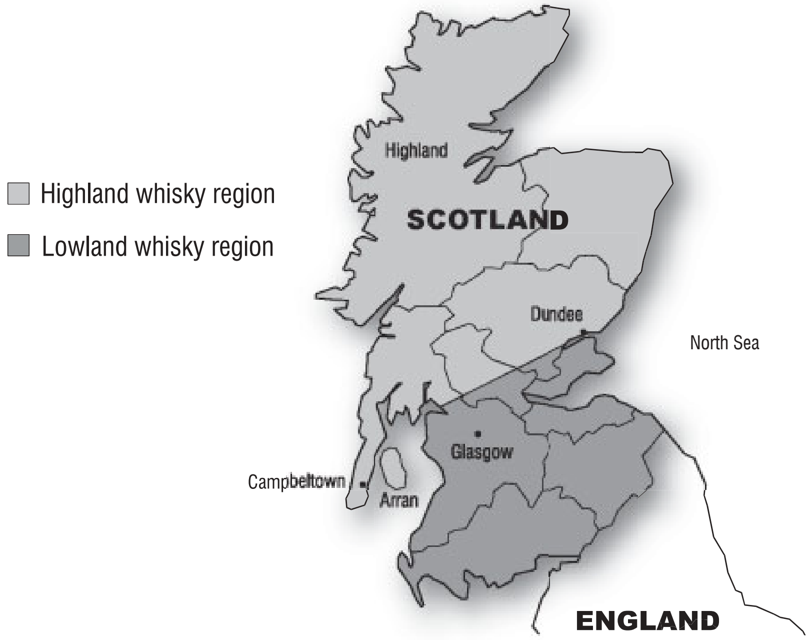


Figure 1. Highland and lowland whisky-producing regions of Scotland.

became increasingly efficient. However, in 1779 there was an important change in the distillery laws. An attempt was made to limit the extent of evasion of spirit duty by prescribing a minimum revenue to be exacted from the owner of each still. The effect of this legislation was dramatic. In 1779, there was said to be 1,152 registered stills in Ireland. By 1790, this number had fallen to 216 and this inevitably fostered widespread illicit distilling (McGuire, 1973). This legislation lasted until 1823 when it was replaced by laws that taxed Irish whiskey on the volume of production, legislation that is essentially still in force today. Development of the Irish whiskey distilling industry in the present century has inevitably been influenced by economic circumstances and by the political division of Ireland into the Republic and Northern Ireland that occurred in 1922. Barnard (1887) described visits to 28 distilleries in Ireland, but closures and amalgamations followed such that when McGuire (1973) prepared his account, there were only two whiskey-distilling companies in Ireland, one with distilleries in Dublin and Cork in the Republic and the other with plants in Bushmills and Coleraine in Northern Ireland. These two companies have since amalgamated and have concentrated their distillery operations in Cork and Bushmills. There has also been a move towards production of a lighter Scotch-type whisky in Ireland to replace the heavier traditional Irish whiskey.

Outline of whisky production processes

Whiskies differ basically in the nature and proportion of the cereals used as raw materials and on the type of still used in the distillation process. These differences in the production process are illustrated in the flow diagram in Figure 2 for production of Scotch malt whisky (production of Irish whiskey is very similar). Detailed accounts of each of the unit processes in whisky production are given in subsequent sections of this chapter.

A characteristic of Scotch malt whisky is that the only cereal used in its manufacture is malted barley (Table 1). After milling, the meal is mashed in a mash tun (Figure 2) similar to that used in breweries for beer production. During mashing or conversion, enzymes in the malt catalyze the hydrolysis of starch into fermentable sugars. In the manufacture of Scotch grain whisky and Irish whiskey, other cereals are used along with malted barley to provide additional starch in the mash tun (Table 1). Owing to the high gelatinization temperature of their starches, unmalted cereals must be precooked before they are incorporated into the mash.

The wort, or clear mash, leaving the mash tun is cooled and fed into a vessel where it is mixed with yeast. In Scotland and Ireland these fermentation vessels have a relatively small capacity and are known as 'washbacks' (Figure 2).

Table 1. Raw materials and unit processes used in the production of Scotch and Irish whiskies.

	Scotch malt	*Scotch grain*	*Irish*
Raw materials	Peated and unpeated malted barley	Wheat or corn and a small proportion of malted barley	Unmalted barley and unpeated, malted barley
Conversion	Infusion mash	Mash cook followed by conversion stand	Infusion mash
Fermentation	Distillers yeast and brewers yeast	Distillers yeast	Distillers yeast
Distillation	Two pot stills	Continuous still	Three pot stills
Maturation	At 62° GL in used charred oak bourbon whiskey barrels or sherry casks for at least three years	Up to 67° GL in used charred oak bourbon whisky barrels or sherry casks for at least three years	At 70° GL in used charred oak bourbon whisky barrels or sherry casks for at least three years

Fermentation is conducted with strains of the yeast *Saccharomyces cerevisiae* that are usually specially propagated for the purpose, although Scotch malt whisky distillers may use some surplus brewers yeast (Table 1). The process is allowed to proceed to a point at which the specific gravity of the fermented mash has usually dropped to below 1.000. In pot still distilleries, the fermented mash or 'wash' (beer) is fed directly to a still known as the wash still, from which the distillates are redistilled in the second or low wines still. In Ireland, and in one Scotch malt whisky distillery, a third distillation is carried out. Finally, the freshly distilled whisky is stored in charred oak barrels for minimum periods of time that depend on the legislation in the producing country (Table 1). Scotch malt and Irish whiskies are customarily matured for much longer than the legal minimum period.

Individual operations

RAW MATERIALS

Malted cereals

Malted barley is the principal malted cereal used in whisky production. Like the brewer, the whisky distiller uses barley cultivars of the species *Hordeum vulgare L.* and *Hordeum distichon* (Hough *et al.*, 1971). Malted barley is employed as a source of enzymes (principally amylolytic) that catalyze the hydrolysis of starches and in some instances serves as a source of starch that is converted ultimately into ethanol. These two demands must be finely balanced. In the manufacture of Scotch malt whisky, where only malted barley is used, care must be taken when the grain is sprouting during the malting process to ensure that only a limited amount of enzyme activity is produced. This is because enzyme is produced at the expense of the fermentable materials in the grain (referred to as 'extract'). However in the manufacture of other types of whisky, malted barley is often used as the only source of amylolytic enzyme in a mash bill that contains a high proportion of unmalted grain. In this type of whisky production the enzyme activity of the malted barley must be greater than that used in Scotch malt whisky manufacture.

Traditionally, barley used in the production of Scotch malt whisky was malted on the distillery premises using a floor malting system and dried over fires of coke and peat in the pagoda-shaped kilns which are still a feature of these distilleries. To a large extent, this system has been superseded by mechanical maltings, which

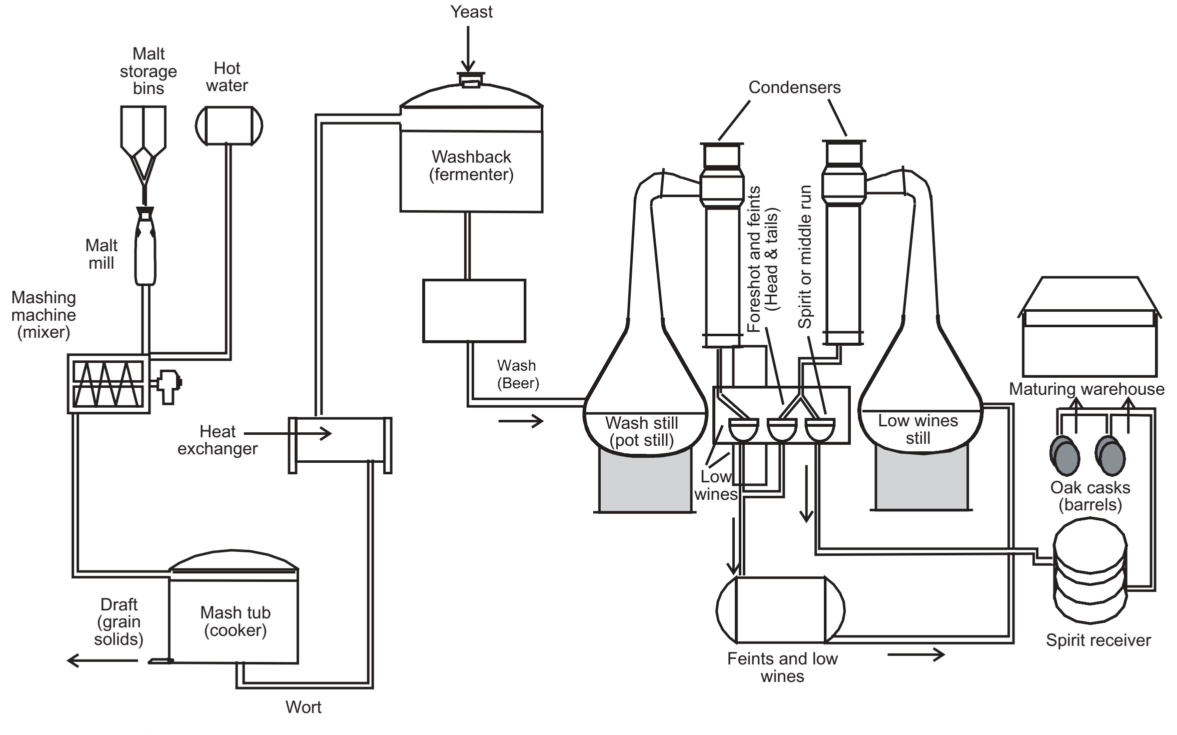

Yeast
Malt storage bins
Hot water
Condensers
Washback (fermenter)
Foreshot and feints (Head & tails)
Spirit or middle run
Malt mill
Mashing machine (mixer)
Wash (Beer)
Maturing warehouse
Heat exchanger
Wash still (pot still)
Low wines still
Low wines
Oak casks (barrels)
Draft (grain solids)
Mash tub (cooker)
Spirit receiver
Feints and low wines
Wort

Figure 2. Flow diagram showing the principal operations during production of Scotch malt whisky.

produce malt for groups of distilleries. In order not to destroy the enzyme activity developed during malting, a balance must be achieved in the kiln between drying the green malt to a suitably low moisture level for storage, curing to give it the appropriate flavor and retaining sufficient enzyme activity (Simpson, 1968). In maltings attached to the distillery, the kiln temperature is increased slowly over a 48 hr period to achieve an even rate of drying and the desired flavor. The latter character is achieved by fuelling the furnace with peat during the early part of the kilning period when the green malt is moist and readily absorbs the peat smoke or 'reek'. In mechanical maltings the green malt is dried at a faster rate with a forced-air draught, but a supplementary peat-fired kiln is often used to produce flavored malts. The amount of peat used varies with different maltings. Some of the distilleries on Islay in Scotland specialize in producing a whisky with a very pronounced peat flavor, and they therefore use heavily-peated malts. Malted barleys used in the manufacture of Scotch grain and Irish whiskies are not dried over a peat fire. They generally have a greater enzyme activity, so the relatively small proportion of malted barley used in the mash contains sufficient enzyme activity to convert all of the starch in the mash (principally supplied by unmalted cereals) into fermentable sugars. The greater enzyme activity in these malted barleys is reflected in their nitrogen content. Malts used in production of Scotch grain whisky have a nitrogen content of 1.8% or higher (compared with a brewer's malted barley with a nitrogen content in the region of 1.5%) (Hough *et al.*, 1971). Malting aids, such as gibberellic acid and bromate, are not normally used in production of malted barley for whisky manufacture.

Because of the high cost of malted cereals, considerable effort has been expended by the whisky distiller in attempting to devise methods that allow prediction of the yield of alcohol expected using different proportions of malted barley in the mash bill. Unfortunately, the methods customarily used by the brewer, such as those recommended in Great Britain by the Institute of Brewing Analysis Committee (1975), have proved of limited value. The brewer has used measurements of diastatic power, expressed as the Lintner value, which is a measure of the extent of saccharification of soluble starch present in a cold water extract of the malt (Lloyd Hind, 1948) as an indicator of malt quality. Diastatic activity measured in this way includes contributions from both α- and ß-amylases. However, Preece and his colleague (Preece, 1947; 1948; Preece and Shadaksharaswamy, 1949 a,b,c) have shown that high ß-amylase activity, as determined by the Lintner value, is not always accompanied by high α-amylase activity. Pyke (1965) showed that the Lintner value of a malt is only useful for predicting the spirit yield in manufacture of Scotch grain whisky when the proportion of

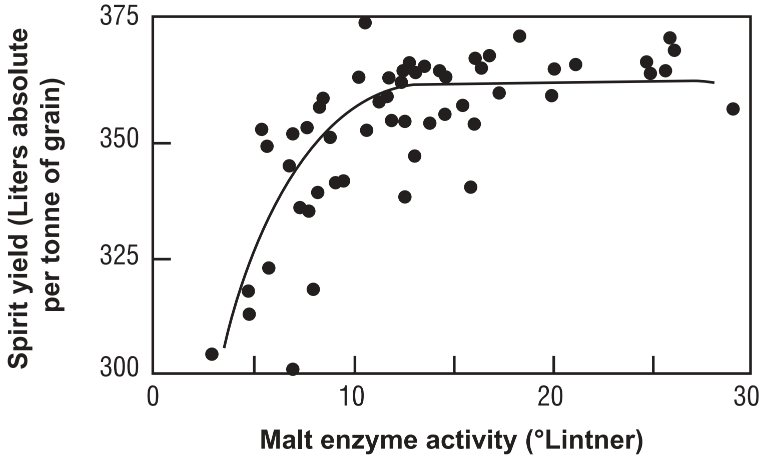


Figure 3. Relationship between the diastatic activity of a Scotch grain whisky mash bill and spirit yield. Laboratory fermentations were conducted using mash bills containing different proportions of malt mixed with corn, and therefore with different Lintner values. It can be seen that only when the proportion of malt is rate-limiting can spirit yield be correlated with the Lintner value (Pyke, 1965).

malted barley in the mashbill is low (Figure 3). Determination of α-amylase activities of the malt gave a less satisfactory correlation than the Lintner value, an observation which agrees with that made earlier by Thorne *et al.* (1945). Further evidence for the unsuitability of employing traditional malt specifications for predicting performance in whisky manufacture has come from Griffin (1972).

Although the degree to which a malted barley has been peated can to some extent be assessed by smell, such is the importance of this character in malt that it must be determined in a more rigorous fashion. Peat smoke or 'reek' contains a wide range of compounds, but it is generally held that the peaty character is imparted to the malt largely as a result of absorption of phenols. For some years, Scotch distillers used a method based on a reaction of phenols with diazotized sulphanilic acid. A lack of specificity in this method, coupled with the instability of the diazonium salt, prompted MacFarlane (1968) to recommend an alternative method involving extraction of phenols from malt with diethylether under acid conditions with absorptiometric measurement of the color developed when the phenols are reacted with 4-aminophenazone. MacFarlane applied the method to a wide variety of malts, both peated and unpeated, and reported values ranging from zero (for an unpeated grain) to as high as 9.4 ppm for a malt produced on Islay in Scotland. (It has been calculated that to obtain a malt with 10 ppm phenols, one tonne of peat must be used for drying each tonne of malted barley).

Scotch whisky distillers have been concerned by the possibility that colorimetric methods, such as those recommended by MacFarlane (1968) and Kleber and Hurns (1972), may not assay all of the organoleptically-important compounds that malt acquires as a result of peating. To examine this possibility, MacFarlane *et al.* (1973) produced peat smoke condensate on a laboratory scale and separated the oil and aqueous phases from the wax fraction, as only the two former would contain components that might appear in the distilled whisky. Six compounds, namely furfural, 5-methylfurfural, guiacol, phenol, ρ-cresol and 5-xylenol, were detected in the aqueous fraction by gas liquid chromatography (GLC). The peat smoke oil was more complex, and no fewer than 30 peaks, some of them created by more than one compound, were obtained by GLC. The compounds included hydrocarbons, furfural derivatives, benzene derivatives and phenols. The authors stressed that using their GLC techniques, 3,5-xylenol is masked by the peaks of m-ethylphenol and ρ-ethylphenol, two compounds thought to make an important contribution to peat aroma and taste. Figure 4 shows gas liquid chromatograms of extracts of a peated and a unpeated malt.

Unmalted cereals

Fewer problems are encountered in arriving at specifications for the unmalted cereals used in whisky manufacture, namely wheat, corn, rye and barley. The corn (varieties of *Zea mays*) used in mash bills for manufacturing Scotch grain whiskies is usually of the yellow dent type, generally obtained from France. Occasionally white corn is used, and it is reputed to give a higher alcohol yield. Corn is a popular grain because it has a high content of starch that is readily gelatinized and converted into fermentable sugars. The US has imposed controls on the quality of corn used for whisky manufacture. In the US there are three grades of corn, with only grades 1 and 2 being approved for spirit manufacture. In Great Britain, on the other hand, the corn used is normally grade 3 on the US scale.

Unmalted barley used in manufacture of Irish whiskey has a quality intermediate to that used for malting and that used for cattle feed. In this way, the maltster can select the best barley available on the market at the time of purchase. For many years, a small percentage (about 5% of the total) of unmalted oats (*Avena spp.*) was included in mash bills for manufacturing Irish whiskey. It was contended that these grains, with their large husks, improved the texture of the grain bed in the mash tun (to assist in straining off the clear wort from the mash solids) and that oats influenced the flavor of Irish whiskey. Whether either or both of these effects were important will probably never be known, for oats are no longer used in the production of Irish whiskey (Court and Bowers, 1970).

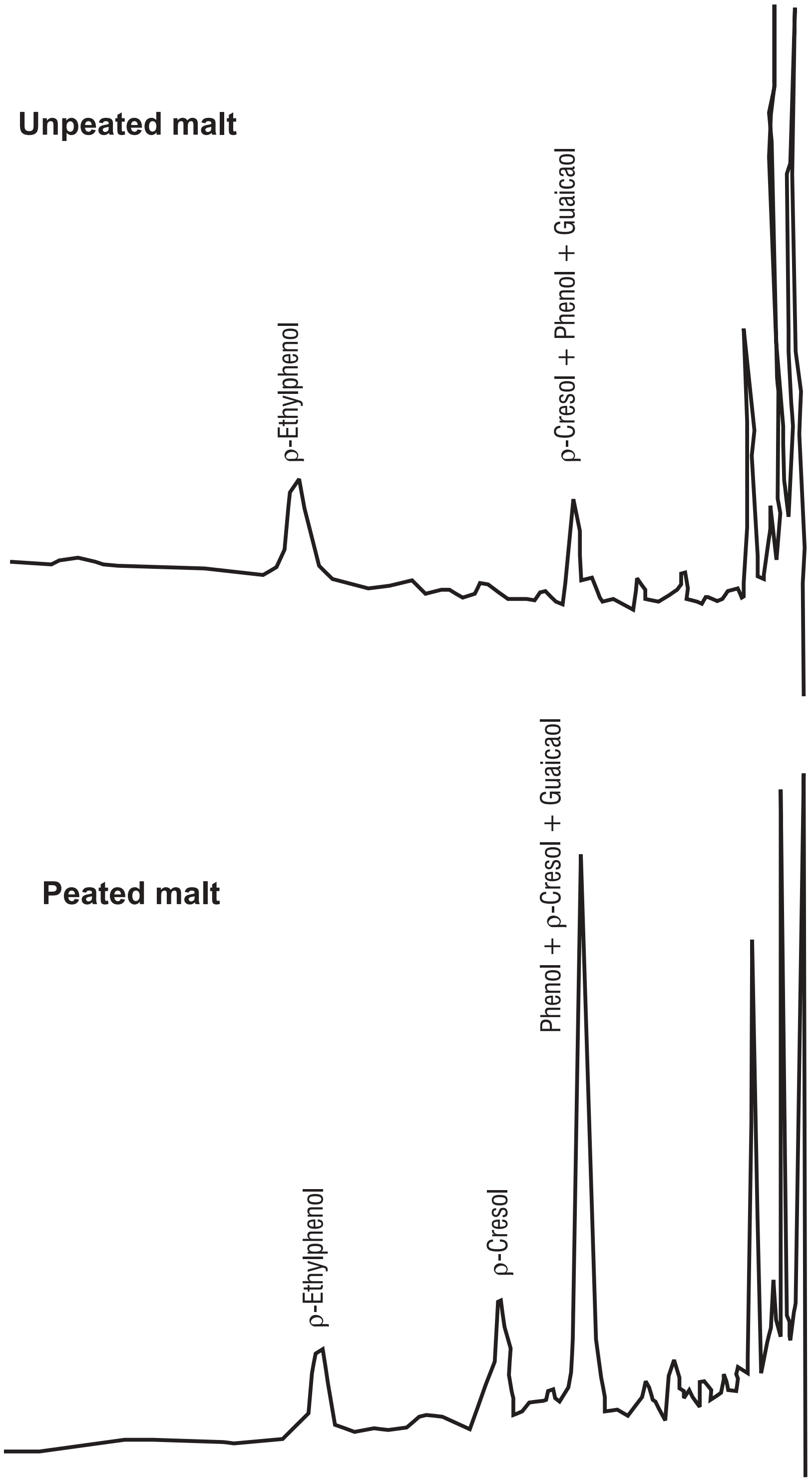


Figure 4. Gas-liquid chromatograms of extracts of unpeated and peated Scoth barley malts showing the contribution peating makes to the content of phenols in the malt. Peating leads to an increase in the size of the peak corresponding to phenol, ρ-cresol and guiacol, and of the peaks which lie to the right of the mixed phenol peak (attributable to furfurals and hydrocarbons). ρ-cresol is also detectable on the chromatogram of extracts of peated malt. The ratio of the area of the total phenol peaks to that of ρ-ethylphenol is used as an indication of the peatiness of the malt.

MASHING AND COOKING

Mash production in Scotch and Irish distilleries involves a process not unlike that used in breweries to prepare wort for beer manufacture. However, where cereals other than malted barley are used, the malt mashing is preceded by a high temperature cooking process.

Mashing

Regardless of whether the mash bill contains cereals other than malted barley, the main biochemical changes that take place during mashing are hydrolysis of starch, protein and other biopolymers in the meal to produce water soluble low molecular weight compounds that form a fermentable substrate (or wort). The major starch-liquefying and saccharifying enzymes are α- and ß-amylases, while the limit dextrins formed by action of amylases on amylopectin are further hydrolyzed by limit dextrinases.

Barley malt used in the manufacture of Scotch malt whisky is coarsely ground in a roller mill adjusted to give a grind no finer than the malt warrants. Too fine a grind can give rise to a 'set mash' that settles on the bottom of the mash tun to block and impede drainage of the liquid (Simpson, 1968). The mash tun is usually preheated with water and the perforated bottom plates are flooded before mashing commences. The meal from overhead bins is mixed with hot water at 60-65ºC in the proportion of one part meal to four parts water, and the mash is homogenized by action of revolving rakes. In the traditional mashing process, the mash is usually loaded into the tun to a depth of about one meter and allowed to stand for about one hour, after which the wort is drained off from under the grain bed. This liquid extract, which has a specific gravity (SG) of 1.070-1.060 (Figure 5) is collected in an intermediate vessel known as an 'underback'. After being cooled to around 25ºC in a heat exchanger, the wort is pumped into the fermentation vessel. The bed of grains in the tun is then re-suspended in water at 75ºC and a second batch of wort is drawn off at a specific gravity of around 1.030 and passed into the underback. This process is known as the first aftermash and is repeated twice more; except that the dilute worts drawn off are not passed to the underback, but are returned to the hot water tank to be used in the next mash. The wort in the underback has a pH value of about 5.5, a specific gravity of 1.045-1.065, and an amino nitrogen content of about 150-180 mg/L. The spent grain residue or 'draff' is removed from the mash tun and sold as animal feed. Many distilleries have now reduced this process to just three water additions. Larger distilleries are now installing semi-lauter tuns in place of the traditional mash tuns. These have vertical knives,

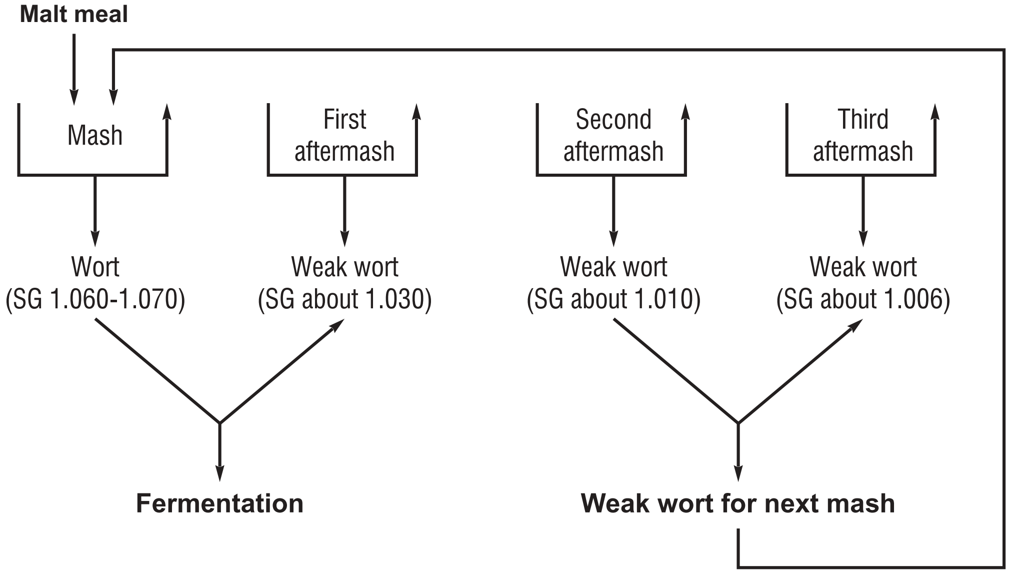


Figure 5. Flow diagram showing the mashing cycle in a Scotch malt whisky distillery.

enabling the structure of the mash bed to be maintained whilst speeding wort draining, and sparging rings to allow simultaneous draining and sparging. The result is a faster more efficient extraction without loss of clarity or changing the composition of the wort.

Mash preparation in the manufacture of Irish whiskey is very similar to that used in Scotch production, but there are certain differences. Use of a high percentage of raw barley in the mash bill (up to 60%) has necessitated the use of stone mills or hammer mills to achieve the required grind. The unmalted barley is sprayed with water to give a moisture content of about 14% and then dried to around 4.5% moisture before grinding. The malted barley used is roller-milled. In recent years a plant has been installed in Ireland which uses a wet milling process that eliminates the need for watering and drying of the unmalted barley. Mash tuns used in Irish whiskey distilleries differ from those used to make Scotch in that they are larger, simply because these distilleries have stills with a greater capacity than those in Scotland. (This difference dates back to when there was a flat rate of tax per still in Ireland, rather than a tax per gallon of product.) Moreover, the mashing cycle differs in that the weak wort from the first aftermash is not passed to the underback, but is mixed with worts from the second and third aftermashes to be used in the subsequent mash (Lyons, 1974).

Cooking followed by malt mashing

The wheat, corn and rye used in production of Scotch grain whisky must be cooked before being added to the mash containing malted barley in order that the starch in these grains can become gelatinized and accessible to the malt enzymes. Traditionally, cooking was carried out as a batch process, but it has to some extent been superseded by continuous processes.

For batch cooking, the grain is freed from extraneous material by passage through screens and then ground either in a pin-type mill or a hammer mill. It is then conveyed to the cooker and subjected to a cooking cycle involving high temperatures and pressures designed to bring about complete gelatinization of the starch. Inadequate cooking will sometimes leave starch granules intact in the mash, while excessive heating can cause caramelization and therefore loss of sugar and decreased spirit yield. Pyke (1965) has provided an account of the cooking of corn in its production of Scotch grain whisky and a similar process is still used today. The conventional cooker is a horizontal cylindrical vessel capable of working at pressures up to 90 psi (63 x 10^4 Pa), and fitted with stirring gear. A typical cycle in cooking corn might consist of 1-1.5 hrs of heating with live steam injection to reach a temperature of 120ºC and a pressure of 15 x 10^4 Pa. The mash is held at this temperature and pressure for a further 1.5 hrs. The liquid used for the mash is often that from the third aftermash mentioned earlier. At the end of the cooking time, the pressure is released and the hot cooked corn mash is blown directly into a saccharification vessel containing the malted barley suspended in hot water. Cold water is then added to bring the temperature in the mash tun to around 63ºC. Good mixing is also essential at this stage; and failure to achieve it can lead to the entire mash solidifying. The need for rapid cooling of the corn mash was emphasized by Murtagh (1970), who showed that with slow cooling or holding at a temperature around 82ºC lipid-carbohydrate complexes could be formed that are not fermentable and can lead to a loss of 1-2% in spirit yield.

After a suitable mashing period, the mash may be filtered as described previously. More commonly, the entire mash may be pumped via a heat exchanger into a fermentor. Pyke (1965) has published the composition of a typical Scotch grain whisky mash (Table 2).

In the manufacture of Scotch grain whisky, the proportion of malted barley in the mash bill is usually around 10-15%, a proportion far greater than that required merely to provide a source of amylolytic enzymes. It is a practice of long standing, and is done to obtain the required malt flavor in the grain whisky. This was noted as early as 1902 by Schidrowitz and prompted further comment from Valaer in 1940.

Continuous cooking has been adopted in recent years in Scotland. Stark (1954) listed the advantages of using continuous cooking. While practices vary to some extent in different distilleries, essentially the cereal is slurried at around 50ºC and then pumped through a steam-jet heater into the cooking tubes where the residence time is normally 5-10 minutes. The continuous cooker has a narrow tube (6-16 cm in diameter) which reduces the incidence of charring and carbon deposition. The mash passes through the tube at about 20 meters/ minute, with the temperature reaching near 65ºC and the pressure 65 psi (40 x 10^4 Pa).

Table 2. Chemical composition of a typical scotch grain mash*.

	%
Total soluble carbohydrate (as glucose)	9.00
Insoluble solids	2.20
Fructose	0.13
Glucose	0.29
Sucrose	0.28
Maltose	4.65
Maltotriose	0.96
Maltotetraose	0.45
Dextrin	2.54
Amino nitrogen (as leucine)	0.09
Ash	0.27
containing P_2O_5	0.09
K_2O	0.09
M_gO	0.02
	µg/ml
Thiamin	0.46
Pyridoxine	0.61
Biotin	0.01
Inositol	236
Niacin	11.1
Pantothenate	0.71

*Pyke, 1965.

Fermentation

The objectives in fermenting a whisky distillery mash with strains of *Saccharomyces cerevisiae* are to convert the mash sugars to ethanol and carbon dioxide while producing quantitatively minor amounts of other organic compounds which contribute to the organoleptic qualities of the final distilled product. Fermenting vessels vary considerably in volume, depending on the distillery. The small Scotch whisky distillery at Edradour near Pitlochry, for example, has fermentors with a capacity of only 4,500 liters. In contrast, other Scotch and Irish pot whiskey distillers have fermentors with a capacity in the range 50,000 to 150,000 liters. Much larger fermentors are found in Scotch grain whisky distilleries. Traditionally, the smaller pot distillery fermentors were made of wood, usually of larch or Oregon pine, but in recent years they have been constructed of steel or aluminum. In some Scottish distilleries, timber is still used as a covering material.

When the fermentor is partly filled, the mash is inoculated with a suspension of *Saccharomyces cerevisiae*. The source of yeast varies with the location and size of the distillery. Distilleries in Scotland and Ireland, particularly the pot whisky distilleries, rarely have their own yeast propagation equipment. They rely on specially-propagated pressed yeast for use in fermentations. In Scotch malt whisky distilleries, this pressed yeast is augmented, usually on an equal weight basis, with surplus brewery yeast typically supplied in compressed form from breweries that may be as far as 500 km from the distillery.

The requirements for a strain of *Saccharomyces cerevisiae* yeast used in distillery practice have been described by Harrison and Graham (1970). Apart from the obvious need to select a strain that maintains very high viability in the pressed state (containing 25% dry weight), other very important properties of these strains are ability to tolerate concentrations of ethanol of the order of 12-15% (v/v) and the capacity to metabolize oligosaccharides such as maltotriose and maltotetraose in order to maximize the conversion of starch into ethanol and carbon dioxide.

Whisky worts usually have a specific gravity in the range 1.050-1.080, a pH value of around 5.0, a total acid content of 0.1% and an optical rotation of +30º. After inoculation, the yeast content is 5-20 million cells/ml. The bacterial count varies with the cleanliness of the plant and the extent to which the raw materials were endowed with a microbial flora. Scotch grain whisky fermentations create little if any foam because of their large content of suspended solids. However, in most Scotch malt whisky fermentations only a small proportion of the suspended solids in the mash is retained in the fermentation vessel. These fermentations tend to foam and the distillers have resorted to the use of various types of antifoams.

Changes over the time course of a typical fermentation in a Scotch malt whisky distillery are depicted in Figure 6. Fermentation proceeds vigorously for the first 30 hrs, during which time

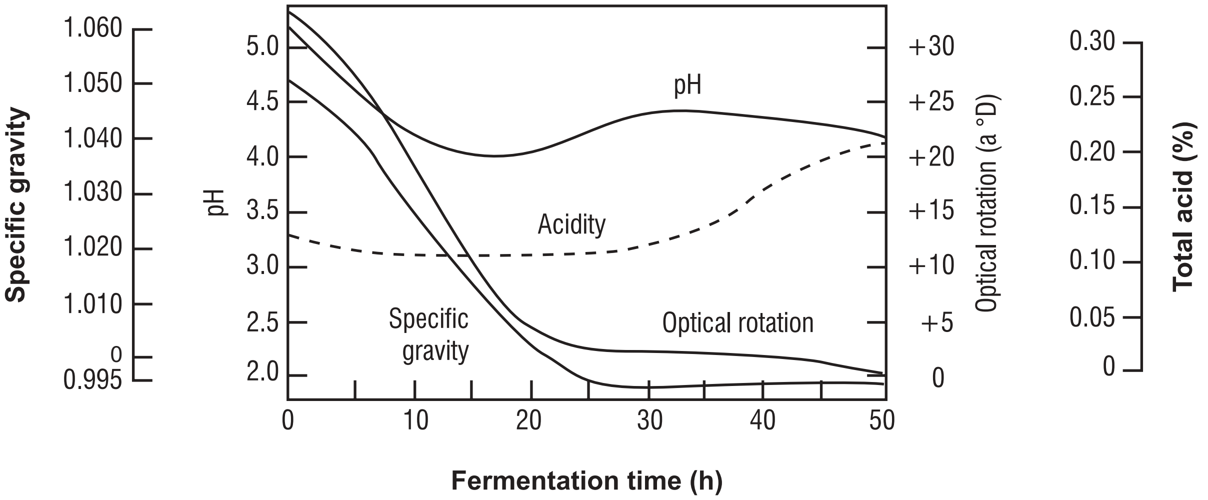


Figure 6. Changes in specific gravity, optical rotation, pH value and acidity during fermentation of a mash in the production of Scotch malt whisky (Dolan, 1976).

the specific gravity falls to 1.000 or below and the optical rotation to around zero. The sugars in the wort are utilized in a particular sequence with glucose and fructose being fermented first, followed by maltose and then maltotriose. The removal of sugars during fermentation of a Scotch grain whisky mash is shown in Figure 7 (Pyke, 1965). Over the first 30 hrs the pH value, after declining to around 4.2, rises to about 4.5. During the first 30 hrs the specific gravity drops at a rate of about 0.5° per hour accompanied by a massive evolution of heat. While many of the larger grain whisky distilleries have fermentors fitted with cooling coils, these are absent, or if

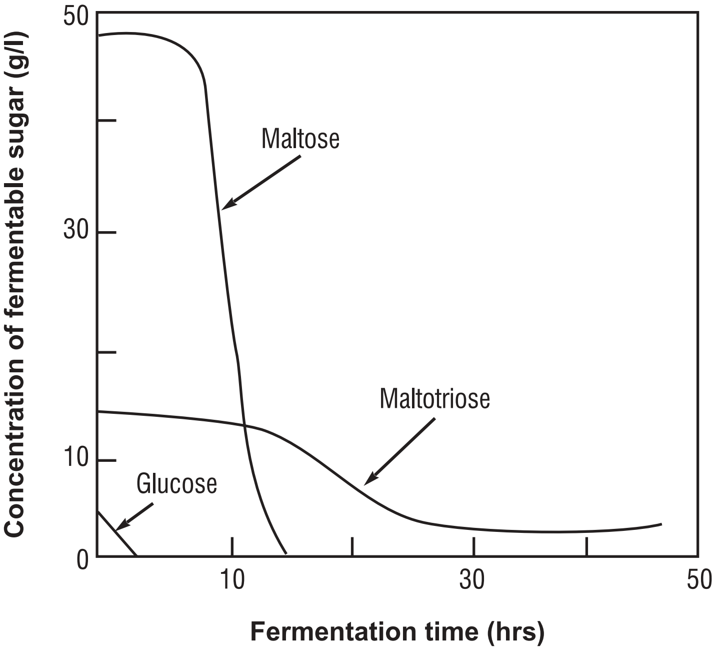


Figure 7. Time course removal of fermentable sugars from a Scotch grain whisky mash (Pyke, 1965).

fitted are relatively inefficient in most malt whisky distilleries where temperature can rise by the end of the fermentation to as high as 35-37°C. The distiller is concerned about the temperature rise during fermentation since this can cause the fermentation to stop or become 'stuck'. Temperature rise can be controlled by using a lower starting temperature or, because glycolysis of sugar is a heat-producing process, by using a lower initial concentration. Strains of *Saccharomyces cerevisiae* are well suited for malt whisky distillery fermentations since they can ferment efficiently over a wide temperature range. Fermentation is usually continued for at least 36 hrs and frequently longer, at which time the ethanol content of the wash is 7-11% (v/v). In larger distilleries, particularly those in the US, the carbon dioxide evolved is collected, liquefied and sold. Smaller distilleries, particularly the malt whisky distilleries in Scotland, usually do not have this facility.

It should be noted that mashes in malt whisky distilleries are not boiled, so any enzyme activity manifested at the temperature of the mash and any microorganisms that can survive at that temperature will continue to be active during the fermentation. The continued activity of limit dextrinases in unboiled distillery mashes increases the concentration of sugars available for fermentation by the yeast. Hopkins and Wiener (1955) calculated that with amylases alone the yeast cannot metabolize the equivalent of the final 12-16% of the starch.

Another important consequence of using non-sterile conditions in distillery fermentations is the activity of bacteria that pass through in the mash, which are encouraged to some extent by the relatively high temperatures to which the fermentations can rise. In addition to lactic acid bacteria, the flora can include other Gram-positive as well as Gram-negative strains. The concentration of the flora depends on a number of factors including the extent to which the lactic acid bacteria grew during yeast propagation, the extent of the flora on the cereal raw materials and on the standard of hygiene in the distillery. There is no doubt, however, that the controlled activity of this bacterial flora, and particularly of the lactic acid bacteria, is accompanied by excretion of compounds that contribute to the organoleptic qualities of the final whisky (Geddes and Riffkin, 1989).

During the first 30 hrs or so of malt whisky fermentation there is vigorous fermentation and the majority of the aerobic bacteria die. This, however, provides ideal conditions for growth of anaerobic or microaerophilic bacteria, principally lactic acid bacteria (mainly strains of *Lactobacillus brevis, L. fermenti* and *Streptococcus lactis*) with the result that the concentration of lactic acid in the fermented mash can be as high as 30 mg/L (MacKenzie and Kenny, 1965). A wide range of lactobacillus species have been identified in Scotch whisky fermentations including *L. fermentum, L. brevis, L. delbrueckii, L. plantarum, L. casei* and a bacterium resembling *L. collinoides*, in addition to *Leuconostoc spp.*, *Streptococcus lactis* and *Pediococcus cerevisiae* (Bryan-Jones, 1976). More recently Barbour (1983) isolated many species that did not conform to recognized species of lactic acid bacteria, a point emphasized by Walker *et al.* (1990) who used DNA hybridization techniques to classify distillery bacteria. Growth of lactic acid bacteria is probably enhanced by yeast excretion of nitrogenous nutrients at the end of a vigorous fermentation. Kulka (1953) demonstrated the ideal nature of yeast autolysate for growth of lactobacilli. Bacterial activity in the fermenting wort also leads to removal of some acids. Actively growing yeasts secrete citric and malic acids, but MacKenzie and Kenny (1965) attribute the lower concentrations of these acids in malt distillery worts (as compared to brewery worts) to their partial removal by bacteria.

Occasionally, the extent of the bacterial flora in the fermenting wort can become too large. This causes problems due to sugar utilization by the bacteria that lead to an overall decrease in spirit yield. In addition, the bacteria may produce organoleptically-undesirable compounds and also release hydrogen ions causing the pH value of the wort to fall too low, thereby providing suboptimal conditions for action of certain enzymes. Examples of undesirable compounds that may be excreted by bacteria are hydrogen sulfide and other sulfur-containing compounds (Anderson *et al.*, 1972). Lactobacilli can also metabolize glycerol (excreted by the yeast during fermentation) to produce ß-hydroxypropionaldehyde, which subsequently breaks down on distillation to give acrolein (Harrison and Graham, 1970). Acrolein imparts a pungent, burnt and often peppery odor to the whisky (Lyons, 1974). In a later paper, Dolan (1976) concentrated on the problems arising in malt whisky distilleries when there is an unacceptably high concentration of bacteria in the mash. Table 3 shows changes in the concentrations of Gram-negative and Gram-positive bacteria and (separately) of lactobacilli during fermentation of a minimally-infected mash and of a heavily-infected mash. The time course of fermentation of an unacceptably-infected malt distillery mash (Figure 8) shows, in comparison with similar data for fermentation of an acceptable mash (Figure 6), a greater rise in the acid content of the mash after about 35 hrs and a lower optical rotation of the mash after about 40 hrs. In the fermentations there is often a difference of up to 4 hrs from the time a rise in the acid content is detected to the point when the pH value of the fermentation begins to fall. Dolan (1976) attributes this to the buffering capacity of the mash. The data in Table 4 show the effect of different levels of infection after 30 hrs fermentation of a malt distillery mash on spirit yield and the associated financial losses to the distiller. Dolan (1976) recommends upper limits of 1,500 bacteria, 50 Gram-positive and 10 lactic acid-producing bacteria per million yeast cells in the mash at the start of fermentation.

Table 3. Changes in the concentration of bacteria during fermentation of a minimally infected and a heavily infected Scotch malt whisky wort.*

	Minimally-infected wort		*Bacteria/ml*		*Heavily-infected wort*	
Age (hours)	*Gram-negative rods*	*Gram-negative cocci*	*Lactobacilli*	*Gram-positive rods*	*Gram-positive cocci*	*Lactobacilli*
At setting	2,000	3,700	n.d.	52,000	60	0.6 x 10^6
10	150	n.d.	n.d.	n.d.	n.d.	2.3 x 10^6
20	n.d.**	n.d.	1.53 x 10^6	n.d.	n.d.	18.8 x 10^6
30	n.d.	n.d.	10.2 x 10^6	n.d.	n.d.	96 x 10^6
40	n.d.	n.d.	10.2 x 10^6	n.d.	n.d.	502 x 10^6
50	n.d.	n.d.	50 x 10^6	n.d.	n.d.	1 x 10^9

*Dolan, 1976.
**None detected.

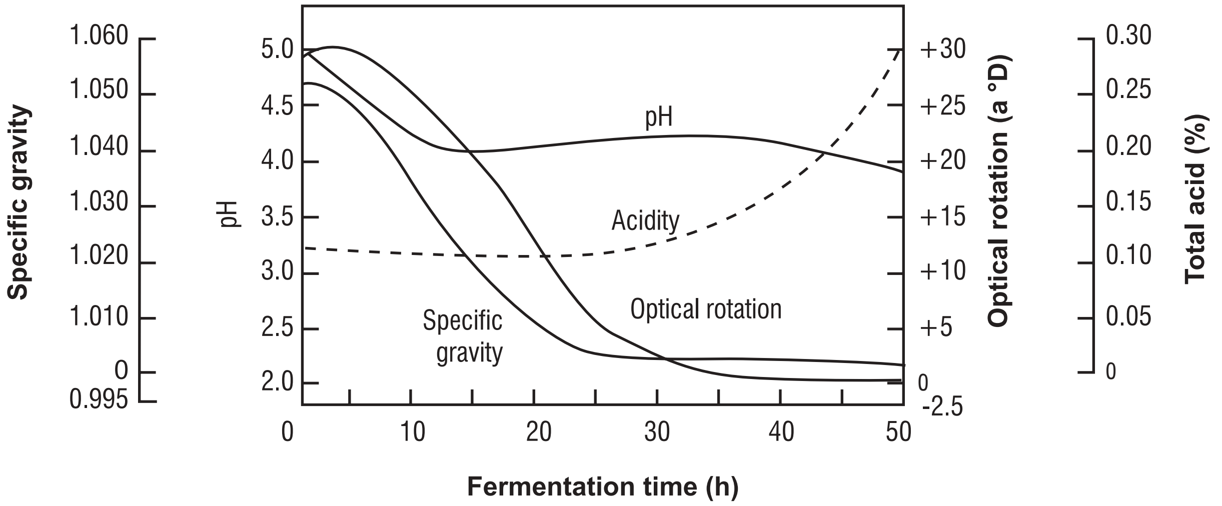


Figure 8. Changes in specific gravity, optical rotation, pH value and acidity during fermentation of a Scotch malt whisky mash containing an unacceptably high concentration of bacterial infection (Dolan, 1976).

Table 4. Effect of the level of bacterial infection on the loss of spirit incurred following fermentation of a Scotch malt whisky mash.*

Infection rating	*Bacteria/ml in 30 hr old mash (million)*	*Approximate loss in spirit yield (%)*	*Approximate financial loss in US dollars (thousands)*	
			At filling	Duty paid
a	0 - 1	<1	<26	<369
b	1 - 10	1-3	26 - 79	369 - 1106
c	10 - 100	3-5	79 - 131	1106 - 1844
d	>100	>5	>131	>1844

*Dolan, 1976.

Much less has been published on the effect of retaining solid material in the fermenting mash. However, marine microbiologists have long known that the presence of solid particles in a liquid medium can affect bacterial growth, probably because of the concentration of nutrients at the solid-liquid interface (Heukelekian and Heller, 1940; Zobell, 1943). Moreover, Cromwell and Guymon (1963) found that formation of higher alcohols during fermentation of grape juice is stimulated by the presence of grape skins or inert solids. Beech (1972) made similar observations on cider fermentations. Merritt (1967), in the only detailed report on the role of solids in whisky distillery fermentations, states that a dry solid concentration of 50 mg/100 ml might typically be expected, although much will clearly depend on the design of the mash tuns used in individual distilleries. Merritt went on to report that a concentration of dry solids as low as 5 mg/100 ml causes an increase in yeast growth, and that solids also enhance the rate of production of ethanol and glycerol. There was also an effect on production of higher alcohols by the yeast (Table 5). With the possible exception of n-propanol, production of all of the major higher alcohols was increased in the presence of solids, the effect being particularly noticeable with isobutanol and 2-methylbutanol.

The effect of low insoluble solids content is a factor relevant to congener levels in malt whisky fermentations. In grain whisky production, where ‘all-grains-in’ fermentations are generally used, the degree of rectification during distillation is the principle determinant of higher alcohol levels in the spirit.

DISTILLATION

Whether the fermented beer is distilled in a pot still, as in production of Scotch malt and Irish whiskies, or in a continuous still based on the Coffey design as in the manufacture of Scotch grain whiskies, the objectives are the same: selective removal of the volatile compounds (particularly the flavor-producing congeners) from the non-volatile compounds and to create additional flavor-producing compounds as a result of chemical reactions that take place in the still. Nevertheless, it is still most convenient to discuss whisky distillation under the separate headings of pot still and continuous distillation.

Table 5. Production of higher alcohols (mg/100 ml).

Mash	*Insoluble solids content (mg/100 ml)*	*n-Propanol*	*Isobutanol*	*2-methyl butanol (amyl alcohol)*	*3-Methyl butanol (isoamyl alcohol)*	*Total*
1	0	1.5	4.2	3.0	8.0	16.7
	25	1.5	6.5	4.4	8.8	21.2
2	0	2.8	4.5	2.7	9.5	19.5
	35	3.3	6.6	3.7	10.1	23.7
3	0	1.7	4.8	3.1	8.0	17.6
	50	1.7	6.8	4.6	9.5	22.6

Pot still distillation

The copper pot still, which is the feature dominating any Scotch malt or Irish whiskey distillery, has changed hardly at all over the centuries, except of course in size. Traditionally, the onion-shaped stills were fired from beneath and had the vapor pipe or 'lyne arm' from the still projecting through the distillery wall to connect with a condenser in the form of a coil immersed in a water tank fed from a local stream (Figure 9). Internal steam-heated calandria are now preferred to direct firing because this decreases the extent of pyrolysis of the still contents and results, for example, in a lower concentration of furfural in the whisky. Variations in still design include expansion of the surface area of the column into a bulbous shape, water jacketing and return loops from the first stage of the condensation. (Nettleton (1913) provided a valuable account of early still design). In many distilleries, condenser coils have been replaced by tubular condensers that have an advantage in that they are designed to conserve the heat extracted from the distillates. Yet other pot stills are fitted with 'purifiers,' which consist of a circular vessel cooled by running water interposed between the neck of the still and the condenser. In Irish pot stills this purifier function is effected by a trough fitted around the lyne arm through which running water is circulated. Pot stills in Scotch and Irish distilleries are traditionally constructed of copper. The reason for this adherence to copper is more than tradition. It has been established that copper fixes some volatile sulfur-containing compounds that are produced during fermentation but undesirable in the distilled spirit.

Early whisky distillers realized that although the objective of distilling was to separate volatile constituents from the beer, collecting the distillate not as a whole but in several fractions and combining certain of these fractions gave a much more acceptable product. Pot still distillation in Scotland and Ireland differ not only in the size of the stills (25,000-50,000 liters in Scotland vs 100,000-150,000 liters in Ireland), but also in the different ways in which fractions are collected from the stills.

In Scotland, the beer is subjected to two distillations. In the first, carried out in the beer still, the beer is brought to a boil over a period of 5-6 hrs and the distillate is referred to as low wines. This distillation effects a three-fold

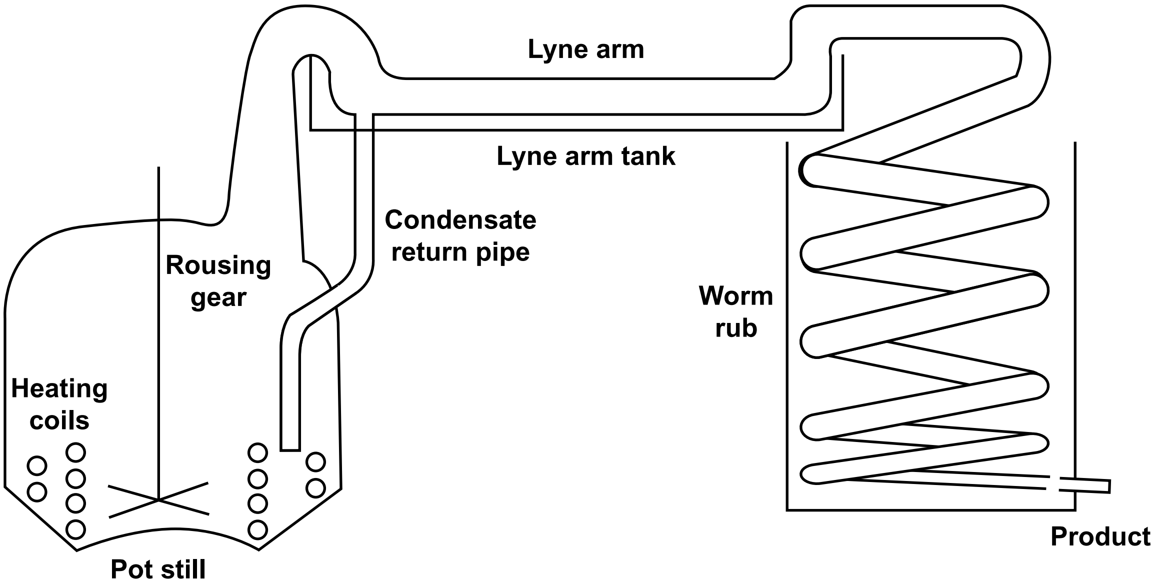


Figure 9. Diagram of an Irish distillery pot still. Designs used in Scotch malt whisky distilleries are similar except that the pot is onion-shaped and the still usually has a shorter lyne arm not surrounded by a lyne-arm tank (Lyons, 1974).

concentration of the alcohol in the beer (Figure 10). The residue in the wash still, known as 'pot ale', is either discharged to waste or evaporated to produce an animal feed (Rae, 1967). Distillation of the low wines in the spirit is still more selective. The first fraction, which contains low boiling point compounds, is rejected as 'fore-shot heads'. At a stage determined by continued hydrometric monitoring, which usually occurs when the distillate has an alcohol content of approximately 70-73° GL, the distillate is switched from the fore shots tank to the whisky receiver tank. This switch has traditionally been made at the discretion of the distiller; and he has been aided by the disappearance of a bluish tinge when water is added to the distillate. The monitoring process takes place in a spirit safe secured with a Government Excise lock. Collection of the distillate is terminated when the alcohol content has fallen to a specified value, although distillation of the feints or tails is continued until all of the alcohol has been removed from the low wines. The residue remaining in the spirit still is known as 'spent lees', and like pot ale is either run to waste or evaporated to manufacture animal feed. The whisky distilled over in the middle fraction has an alcohol content of 63-70° GL.

The manufacture of Irish whiskey involves three rather than two distillations (Figure 11). The fermented beer is heated in the wash still, and the first distillation ('strong low wines') is collected until the distillate reaches a predetermined specific gravity. The distillate, then known as 'weak low wines', is switched into a separate vessel. The weak low wines are pumped into the low wines still and are re-distilled to produce two fractions termed 'strong feints' and 'weak feints'. Strong feints are mixed with the strong low wines in a spirit still. Distillates from this are collected in the same fashion as in production of Scotch. The whiskey collected usually is about 89-92° GL.

Continuous distillation

No fundamental changes have been introduced into the design of the patent or Coffey still over the past century. Automation, particularly of the beer feed, is now commonplace, as is continuous monitoring of other stages in the distillation process. Nevertheless, many Scotch and Irish

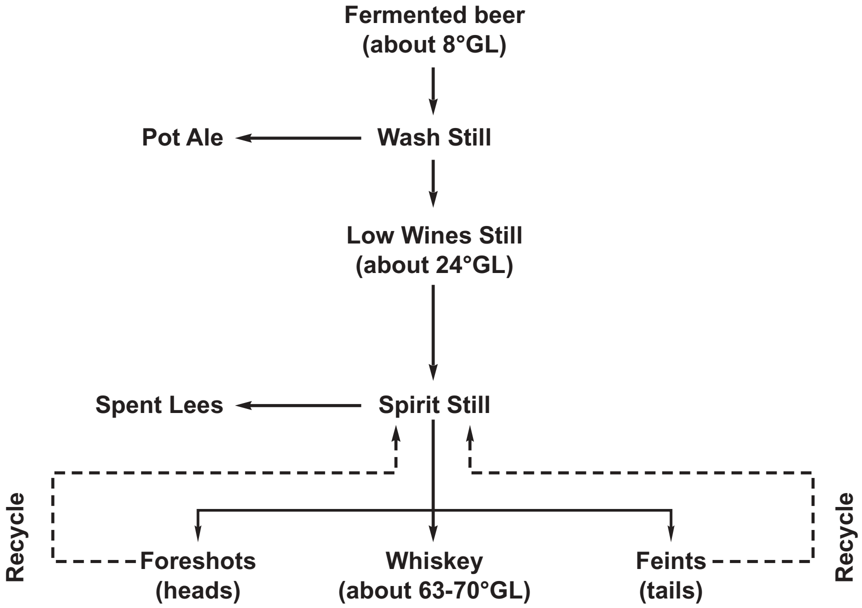


Figure 10. Flow diagram showing the stages in distillation of Scotch malt whisky.

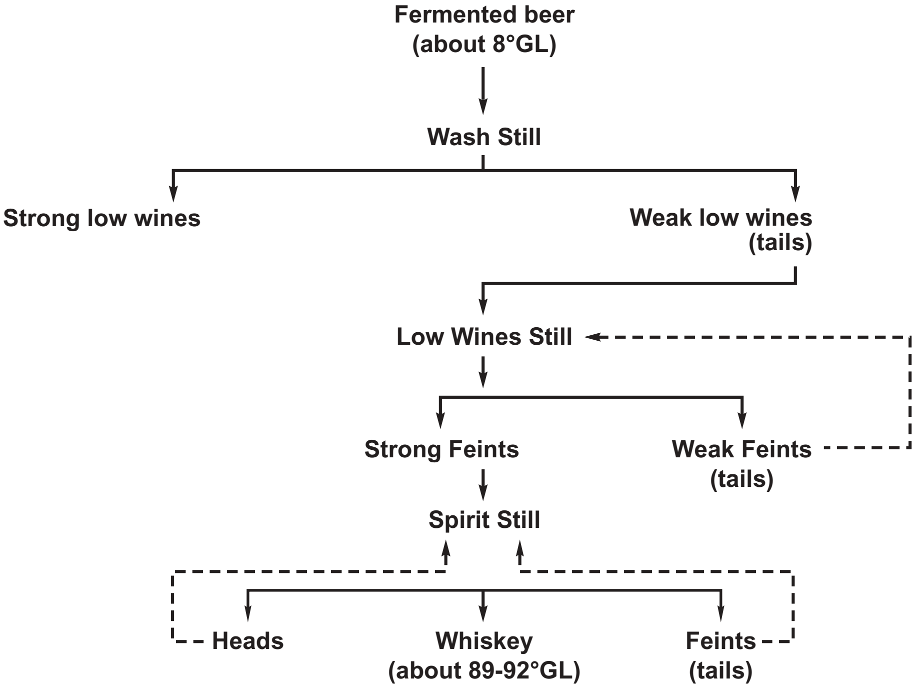


Figure 11. Flow diagram showing the stages in distillation of Irish whiskey.

producers of grain whiskies continue to use a still which, like the original Coffey still, has just two columns: a beer stripper (or analyzer) and a rectifier.

A description of the operation of two column continuous stills in the manufacture of Scotch grain whisky has come from Pyke (1965). In order to obtain whisky of high quality from these stills, they must be operated such that the alcohol concentration of the spirit at the spirit draw tray in the rectifier is not less than 94.17° GL. The manner in which the precise control of still operation can affect the composition of the whisky is shown in Figure 12. As illustrated, if conditions are changed in either direction on the abscissa, the concentration of congeners will alter with a possible adverse effect on final product quality.

MATURATION AND AGING

Freshly distilled whisky of any type is very different from the spirit that is later bottled, either singly or blended. The transformation is brought about by storing the whisky in oak barrels for periods of time that depend on traditional practice and legal requirements. In general, whiskies are matured for far longer than the legally-required period of time. The raw spirit is taken by pipeline from the distillation plant to the tank house where it is diluted with water to the required strength and then transferred into barrels.

Maturation in barrels is accompanied by a loss of liquid by evaporation, and the relative rates of loss of water and of alcohol determine whether the aged whisky has a higher or lower alcoholic strength than that at filling. In Scotland, where the barrels of whisky are stored in cool, unheated, but humid warehouses, the alcoholic strength decreases (Valaer, 1940). In contrast Valaer and Frazier (1936) reported that in the US storage conditions cause an increase in alcoholic strength. Maturation in barrels is also accompanied by changes in the chemical composition of the whisky. These changes are attributable to extraction of wood constituents from the barrel, oxidation of components present in the original whisky as well as those extracted

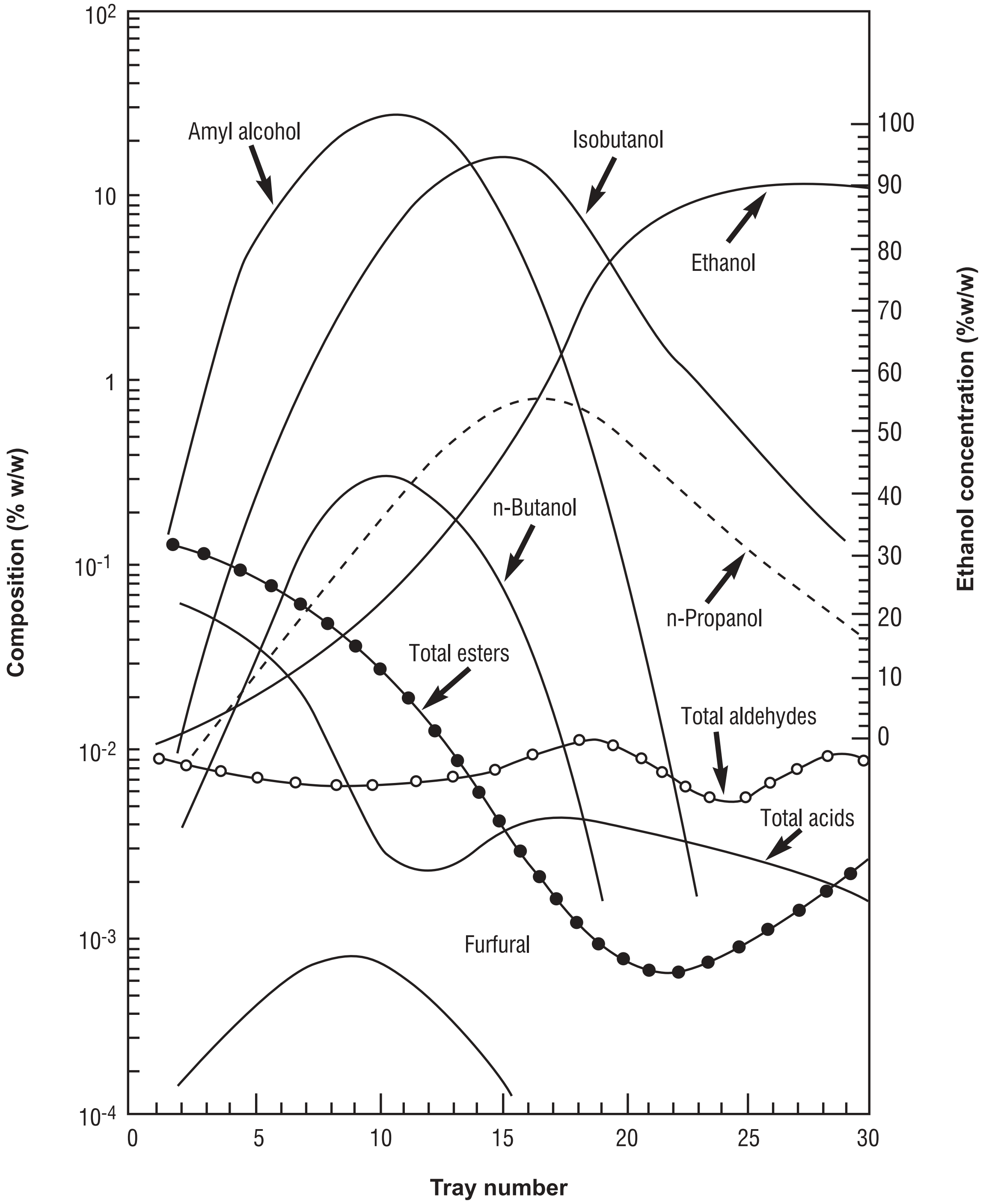


Figure 12. Changes in composition of the vapor at different trays in a Coffey still rectifier used in the manufacture of Scotch grain whisky (Pyke, 1965).

from the wood, reactions between components in the whisky and removal and oxidation of highly volatile sulfur components by the carbon char on the inner surface of the barrel.

Some of the earlier investigators reported on changes in the composition of the major classes of organoleptically-important compounds during maturation in barrels. Thus, Schidrowitz and Kaye (1905) found increased concentrations of volatile acids in aged whiskies, a trend also described in the report of the Royal Commission on Whisky and Other Potable Spirits (1909). Several reports followed. Liebmann and Scherl (1949), for example, reported increased concentrations of acids, furfural, tannins and color with maturation in barrels. The arrival of the gas liquid chromatograph and HPLC greatly accelerated research on this topic; and more recent data on chemical changes that take place during maturation are described later in this chapter.

Maturation of whisky in oak barrels is an expensive process; and it is hardly surprising that consideration has been given to methods for acceleration. Jacobs (1947) described several of these methods including pretreatment of the beer with activated carbon, chemical treatment of the whisky to convert aldehydes into esters and use of oxidation treatments. Such techniques are not used in the industry, which adheres to the traditional nature of the whisky-producing process.

BLENDING AND COLORING

Whisky manufacturers take great pride in the quality of their straight or blended whiskies and in their ability to maintain the quality of a particular product over the years. Blending is conducted by experts who advise manufacturers on mixing or blending large volumes of whisky after appropriate sniffing and tasting sessions on experimental blends. Blended whiskies are filled into re-used barrels after dilution with water (or not) and stored for a further period of time referred to as ‘marrying’. The water used for dilution is softened or demineralized, since water containing an appreciable concentration of salts can cause hazes in the whisky (Warricker, 1960). After marrying, and dilution if necessary, the color of the whisky may be adjusted to the desired value by adding caramel. Some brownish pigment is extracted from the casks, but this may not be sufficient to provide the desired color. Finally, the whisky is clarified for bottling by filtration through sheets of cellulose (Simpson, 1968). Chill filtration may also be practiced, since it removes tannin material from the whisky and prevents subsequent appearance of haze.

EFFLUENT DISPOSAL AND SPENT GRAINS RECOVERY

Traditionally effluents from whisky distilleries were disposed of in the most convenient manner. Spent grains were retailed, often quite cheaply, to local farmers as animal fodder while pot ale and spent lees were simply discharged into the local sewer, stream or river. This is no longer the case, mainly because of the distiller’s awareness of the nutritional value (largely the protein content) of some of these effluents, and public awareness of environmental problems arising from uncontrolled disposal of effluents into waterways. Simpson has described the production of ‘distillers dark grains’. These processes are widely used to dispose of effluents from whisky distilleries, especially where the traditional methods of disposal are forbidden or uneconomic.

Organoleptically important components of whisky

Modern analytical techniques have enabled major advances in the understanding of the compounds responsible for the organoleptic properties of whiskies. However, the first reports on the nature of flavor-producing compounds in whiskies antedate the era of gas liquid chromatography by nearly half a century. Two publications by Schidrowitz (1902) and Schidrowitz and Kaye (1905), dealing exclusively with Scotch whiskies, reported on the higher alcohol, acid and ester contents of some 50 different brands. They reported analyses of several Campbeltown Scotch malt whiskies. A report by Mann (1911) published a few years later also quoted values for acidity and levels of furfural, aldehydes, esters and alcohols in Scotch whiskies imported into Australia.

CONCENTRATIONS OF ORGANOLEPTICALLY IMPORTANT COMPOUNDS

Since the introduction of GLC into distillery laboratories, several reviews have been published on the composition of the major flavor-producing compounds in whiskies (namely higher alcohols, esters, carbonyl compounds, organic acids, aromatic compounds) and on the identification of individual compounds that make up these fractions. Higher alcohols, which are still routinely determined with GLC using a polar stationary phase (Aylott *et al.*, 1994), are quantitatively the most important. Scotch malt whiskies are richest in higher alcohols, with contents often well over 2 g/L. Free fatty acids are relatively volatile and make a major contribution to the organoleptic qualities of whiskies. Concentrations of acids in some Scotch malt whiskies can be as high as 0.4-1.0 g/L absolute alcohol (Duncan and Philip, 1966). Roughly comparable concentrations of esters are found in whiskies, although those produced with pot stills generally have higher concentrations than those from continuous stills. Ester concentrations in Scotch and Irish pot still whiskies have been reported in the range of 0.27-0.87 g/L absolute alcohol (Valaer, 1940). Lighter whiskies contain lower concentrations of carbonyl compounds, although the concentration varies with the brand. Grain whisky may have as little as 20 mg/l of aldehydes, while in a mature Scotch malt whisky the concentration may be as high as 80 mg/L (Duncan and Philip, 1966).

CHEMICAL NATURE OF ORGANOLEPTICALLY-IMPORTANT COMPOUNDS

Duncan and Philip (1966) reviewed chromatographic and other methods used to separate the various organoleptically important compounds from whiskies. Even though analytical methods such as capillary column gas chromatography linked to a mass selective detector (GC-MS) (Aylott *et al.*, 1994) have developed in terms of sensitivity and discrimination, a major problem in analyzing whisky by GLC is the overwhelming preponderance of ethanol and water. Only one volatile compound, namely isoamyl alcohol, is likely to be present in a concentration exceeding 0.01%; while most of the others are present in concentrations that rarely exceed 50 ppm. Indeed many compounds now understood to have an important impact on whisky flavor are present at ppb levels. Carter-Tijmstra (1986) described a technique for measuring dimethyl trisulfide, a compound with a threshold of only 0.1 ppb present in whisky at concentrations below 50 ppb. Analyses are most conveniently conducted on extracted fractions of the different classes of compounds. When direct analysis of whiskies has been employed, only a limited number of components have been determined (Morrison, 1962; Bober and Haddaway, 1963; Singer and Stiles, 1965).

Recent developments in headspace analysis using trapping and thermal desorption techniques (Pert and Woolfendon, 1986) have enabled analysis of the more volatile components of whisky flavor whilst avoiding interference from high concentrations of other congeners. Headspace analysis using trap and purge techniques would now be the method of choice for measuring highly volatile sulfur compounds such as dimethyl trisulfide.

Some idea of the variety of compounds detected in whiskies came from a compilation of both published and unpublished sources (Kahn 1969, Kahn *et al.*, 1969). Of the some 200 compounds listed there are 25 higher alcohols, 32 acids, 69 esters and 22 phenolic compounds. Undoubtedly, this list could now be extended quite considerably. Of the higher alcohols, isoamyl alcohol and optically-active amyl alcohol predominate, accompanied by lower concentrations of isobutanol and n-propanol. Characteristically, there are usually only low concentrations of n-butanol and secbutanol. The principal organic acid in whiskies is acetic acid, which can account for between 50 and 95% of the total content of volatile acids determined by titration. Of the remaining acids, caprylic, capric and lauric are quantitatively the most important (Suomalainen and Nykänen 1970a). Some of the characteristic flavor and aroma of Irish whiskies may be attributed to somewhat higher concentrations of the odoriferous butyric acid. Compared with other types of whisky, Scotch whisky characteristically contains more palmitoleic acid (and its ethyl ester) than palmitic acid. Suomalainen (1971) has suggested that the typical stearin-like smell of Scotch malt whisky may be attributed to long chain fatty acid ethyl esters. It is not surprising to find that ethyl acetate is the major whisky ester in view of the prevalence of acetic acid in the distillate. Concentrations of 95 mg/L have been detected in a blended Scotch whisky (de Becze, 1967). Other esters such as ethyl caprate are present in much lower concentrations, on the order of 2-10 mg/L. Of the carbonyl compounds, acetaldehyde is the principal component together with a range of other short chain aldehydes. Furfural, with an aroma resembling that of grain, also occurs with as much as 20-30 mg/L in Scotch malt whiskies (Valaer, 1940). Acrolein, a pungent and lachrymatory compound, is also present; and it has been suggested that it may contribute to the 'peppery' smell of whisky.

A variety of other organoleptically important compounds has also been detected in different whiskies, many of which result from maturing the whisky in charred oak barrels. Scopoletin and other aromatic aldehydes, including vanillin, were detected in bourbon by Baldwin and his colleagues (1967). These compounds were previously identified by Black *et al.* (1953) in ethanolic extracts of plain and charred American white oak from which the bourbon whiskey barrels subsequently used for the maturation of Scotch and Irish whiskies are constructed. (It should be noted that the US regulations for bourbon whiskey production require that new charred oak barrels be used for maturation; so there is a continuous supply of once-used bourbon barrels that are shipped to Ireland and Scotland for re-use.) A lactone dubbed 'whisky lactone', also appears in whisky following storage in oak barrels. This compound, ß-methyl-octalactone, was first isolated from Scotch whisky by Suomalainen and Nykänen (1970b). It has since been reported that both cis and trans diastereomers of the compound occur in whisky (Nishimura and Masuda, 1971). Other compounds detected in whisky include phenols (Salo *et al.*, 1976), glycerol and erthritol (Black and Andreasen, 1974), pyridine, α-picoline and various pyrazines (Wobben *et al.*, 1971).

CONTRIBUTION OF COMPOUNDS TO ORGANOLEPTIC PROPERTIES

Published information on compounds responsible for the organoleptic qualities of whiskies is meagre and very largely confined to reports by Suomalainen and his colleagues from the State Alcohol Monopoly in Helsinki, Finland. In order to assess the contributions made by whisky components to the odor of these spirits, Salo *et al.* (1972) concocted a synthetic whisky with components that chromatographic analysis had revealed were present in a light-flavored Scotch whisky. The synthetic whisky was made using 576 g of a mixture of higher alcohols, 90 mg of acids, 129 mg esters and 17.4 mg carbonyl compounds in highly-rectified grain spirit diluted to 34° GL in water that had been ion-exchanged and treated with activated charcoal. This imitation whisky contained 13 alcohols in addition to ethanol, 21 acids, 24 esters and 9 carbonyl compounds. Caramel coloring was used to give it the color of a distilled and matured whisky. Odor thresholds of the individual compounds and groups of compounds were determined as described by Salo (1970). Experienced taste panel participants were easily able to distinguish the imitation whisky from a blended Scotch whisky; but when the concoction was mixed with an equal amount of the Scotch, only 6% correct judgments above chance were made. This suggested that the concentrations of, and interactions between, components of the synthetic whisky were not greatly dissimilar from that in the Scotch used for comparison.

Odor threshold determinations of individual components in the imitation whisky revealed that the contributions made by the mixture of alcohols and acids accounted for only about 10% of the total odor intensity, despite the fact that the alcohols themselves accounted for over 70% of the total concentration of organoleptically-important compounds in the concoction. Esters and carbonyl compounds had a much greater influence, particularly butyraldehyde, isobutyraldehyde, isovaleraldehyde, diacetyl, and the ethyl esters of acetic, caproic, caprylic, capric and lauric acids. Since just three of the most important carbonyl compounds could substitute for the whole carbonyl fraction, there would seem to be considerable homogeneity in the odor contributions made by these compounds. Interestingly, the relative contributions made by the different classes of compounds are not very different from the contributions Harrison (1970) reported that they make towards the taste of beers.

Threshold values can be assessed not only for individual components in whisky, but for the total aroma of the beverage. Salo (1975) examined this by diluting different whiskies with water until the characteristic whisky aroma could only just be recognized. Values for the threshold dilution of several commercial whiskies shown in Table 6 reflect the differences in aroma strength for several different commercial whiskies.

An excellent review of how these characteristics can be evaluated from an organoleptic point of view comes from Jack (2003). Sensory data are only as good as the sample preparation and guidelines were set as applicable to the Scotch whisky industry. It is important that the line between sample preparation and assessment should be minimized since time and the temperature of nosing have a major impact on sensory character. As temperature increases, a greater amount of the flavor volatiles are released, thus altering the sensory profile. Effects of changes in room temperature on flavor scores determined by a sensory panel for a blended Scotch whisky are illustrated in the form of a spider diagram in Figure 13.

ORIGIN OF ORGANOLEPTICALLY IMPORTANT COMPOUNDS

The two main sources of the organoleptically important compounds in whisky are the yeast used to ferment the wort and the charred oak barrels in which the whisky is matured. Suomalainen and Nykänen (1966) fermented a nitrogen-free medium containing sucrose with a strain of *Saccharomyces cerevisiae* and distilled the fermented medium either after removing the yeast by centrifugation or with the yeast remaining in the medium. Gas chromatographic analyses of these distillates are reproduced in Figure 14, which also shows a chromatogram of Scotch whisky for comparison. There is clearly a similarity among the three analyses; although differences, such as the higher proportion of isoamyl alcohol in the distillate from the fermented medium, can be detected. Also worth noting is the greater concentration of ethyl caprate in the distillate from the spent

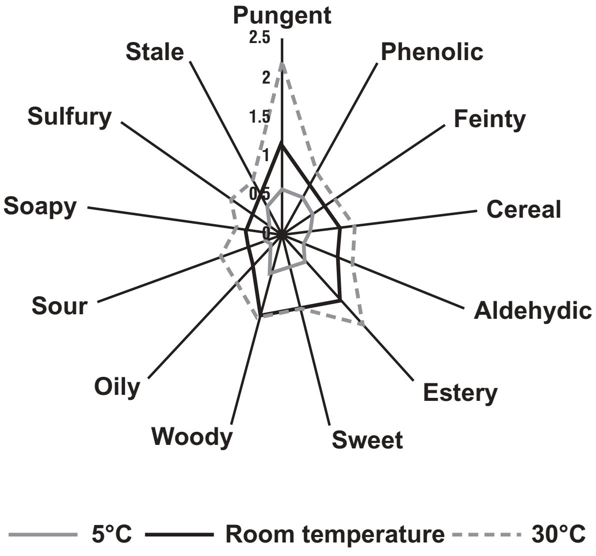


Figure 13. Impact of temperature on flavor profile of Scotch whisky (adapted from Jack, 2003).

Table 6. Threshold dilution levels for nine different types of whisky*.

Whisky	*Threshold dilution (x 10^{-4})*	*One standard deviation range (x 10^{-4})*
Scotch malt	0.56	0.2 - 1.3
Scotch, old blend	0.87	0.5 - 1.5
Scotch, blend	1.20	0.3 - 4.2
Irish	1.30	0.4 - 2.0
Bourbon	2.40	0.6 - 11.5
Irish	4.50	2.7 - 7.5
Canadian	10.40	3.0 - 37.0

*Salo, 1975.

medium containing yeast as compared with that obtained by distilling the medium from which yeast had been removed. It would be interesting to learn of the importance of yeast strain in production of organoleptically-important compounds in whisky. Unfortunately, there is a lack of published data on this matter.

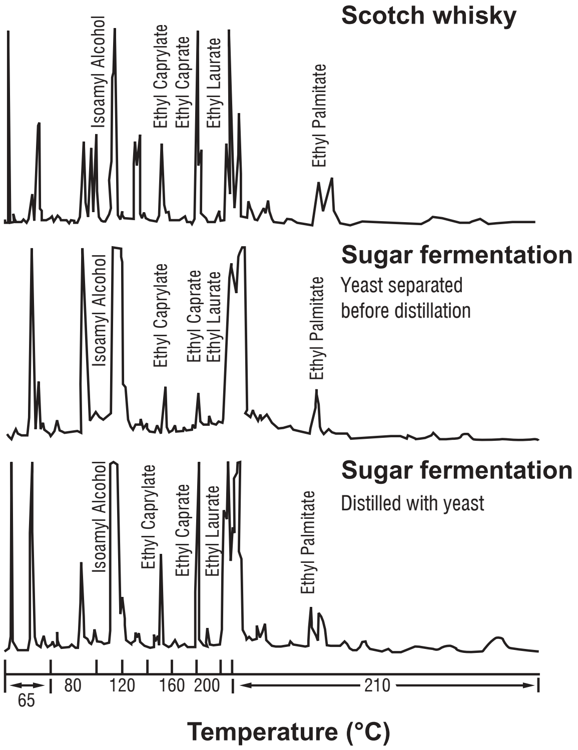


Figure 14. Gas liquid chromatograms of aroma compounds produced by yeast in a nitrogen-free sugar fermentation, with a trace for comparison of the aroma compounds detected in a sample of Scotch whisky (Suomalainen and Nykänen, 1966).

The complexity of the processes for determining the presence and concentration of compounds in whisky may be illustrated by looking at one compound for which this process has been elucidated in detail. In the 1980s ethyl carbamate, a naturally-occurring component of many alcoholic drinks, was identified as being undesirable. Canada specified by regulation a maximum limit for ethyl carbamate in whisky spirit of 150 ppb and the US set guidelines of 120 ppb. Control of this compound was only possible after the processes resulting in its presence in spirit were fully understood. Extensive research in Scotland revealed the mechanism of ethyl carbamate production and facilitated the introduction of very effective control measures. These measures maintain levels close to zero and always less than 30 ppb in distilled whisky spirit.

Cook (1990) reviewed the outcome of this research and the resulting control procedures. When barley sprouts during malting, a glycoside, epiheterodendrin (EPH) is present in the acrospire. This glycoside, which survives kilning and mashing, is extracted into the wort and converted by yeast enzymes to glucose and isobutyraldehyde cyanohydrin (IBAC). The IBAC is stable during fermentation, but when heated above 50ºC at distillation it breaks down to form volatile nitriles. There are a multiplicity of potential routes nitriles can follow including reaction with other beer components or complex reactions often mediated indirectly by copper. Some of the volatile nitriles may escape these reactions and pass into the distillate. A number of reactions can remove the nitriles from the spirit, one being the reaction with ethanol to form ethyl carbamate.

Two control strategies may be used to prevent ethyl carbamate reaching the final spirit. Firstly, some varieties of barley produce low levels of the glycoside EPH (Cook, 1990), and plant breeders are now concentrating on incorporating this character in all varieties for the distilling industry. The second strategy relies on the low volatility of ethyl carbamate. Control of distillation conditions eliminates all the volatile precursor formed by copper mediated reactions. Non-volatile substances are formed prior to the final distillation in malt distillation or before rectification in grain distillation. Thus, ethyl carbamate can be minimized in the final spirit.

A considerable number of studies have been published on those organoleptically important compounds arising either directly or indirectly from the oak barrels in which whisky is matured. The increase in coloring, tannin, dissolved solids and acid concentrations are not observed when whisky is stored in glass, which is proof of the importance of the oak barrels in the maturation process. An analysis of heartwood of the American oak (*Quercus albus*) gave cellulose (49-52%), lignin (31-33%), pentosans (or hemicelluloses, 22%) and compounds extracted with hot water and ether (7-11%; Ritter and Fleck, 1923). However, when charred oak sawdust was directly extracted with water or 96º GL ethanol, the extracts obtained differed markedly in odor from aged whisky. Moreover, none of the various fractions of ethanol-soluble oak extractives contained flavors that resemble mature whisky (Baldwin *et al.*, 1967). As a result, it is now generally held that the maturation process involves not only extraction of compounds from the oak but also chemical modifications of at least some of the compounds extracted from the wood.

For a long time, most of the work reported on this aspect of maturation of whisky came from the laboratories of Joseph E. Seagram and Sons in the US. More recently, accounts of the mechanisms of Scotch whisky maturation have been given by Philip (1989) and Perry (1986) and on the maturation of whisky generally, by Nishimura and Matsuyama (1989). A theme from all this work has been the identification of a number of mechanisms of maturation common to all whiskies. These divide into addition, subtraction and modification by reaction. There is addition of components from the oak wood, including those derived from lignin, tannins and oak lactones. There is the subtraction of volatile compounds from the maturing whisky by evaporation and adsorption on the charred surface of the barrel (Perry, 1986). Lastly there are reaction processes including establishment of equilibria among acetaldehyde, ethanol and acetal (Perry, 1986), polymerization reactions (Nishimura and Matsuyama, 1989) and oxidation-reduction reactions (Perry, 1986; Connor *et al.*, 1990). Many of the reactions involve, and are indeed dependent on, the components extracted from the wood.

Looking at several of these reactions in more detail will serve to illustrate the complexity of the maturation process. The work at the Seagram laboratories, whilst focused on bourbon, is directly relevant to Scotch and Irish whiskies for which once-used bourbon barrels are extensively utilized for maturation. Changes in the concentrations of organoleptically-important compounds during a 12 year storage of a 109º US proof (54.5º GL) bourbon, on a 100º proof (50º GL) basis, are shown in Figure 15. The nature and origin of some of these compounds have been examined in some detail. Among the aldehydes, scopoletin and the aromatic aldehydes syringaldehyde, sinapaldehyde, coniferaldehyde and vanillin are important. According to Baldwin *et al.* (1967), these compounds could be formed by ethanol reacting

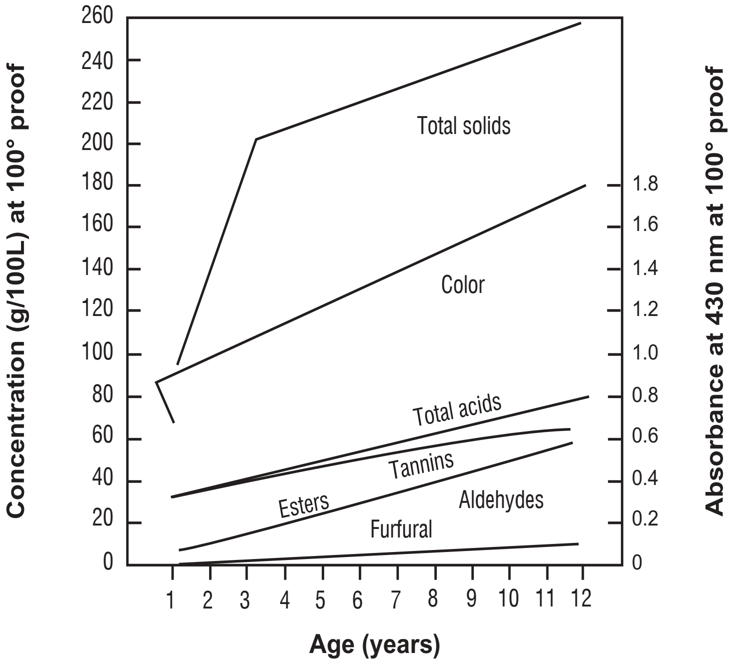


Figure 15. Changes in composition of the vapor at different trays in a Coffey still rectifier used in the manufacture of Scotch grain whisky (from Pyke, 1965).

with lignin in the oak wood to produce coniferyl alcohol and sinapic alcohol. Under mildly oxidizing conditions, these alcohols could be converted into coniferaldehyde and sinapaldehyde, respectively. Vanillin could then arise from coniferaldehyde, and syringaldehyde from sinapaldehyde. The increase in aldehyde content during maturation is also attributable in part to formation of acetaldehyde by oxidation of ethanol. Formation of ethyl acetate probably accounts for the steady rise in the ester content of whisky during maturation.

Several other groups of compounds not described in Figure 14 are also important in the maturation process. Monosaccharide sugars are found in mature whisky, and probably arise from the pentosans and other polysaccharides in the oak wood. Otsuka *et al.* (1963) reported that a mature Japanese whisky contained xylose, arabinose, glucose and fructose, while Black and Andreasen (1974) added rhamnose to this list when they analyzed a mature bourbon. The latter workers found that the concentrations of arabinose and glucose increased at a faster rate than those of xylose and rhamnose over a 12 year maturation period. Salo *et al.* (1976) also detected low concentrations of mannose and galactose in a matured Scotch malt whisky in addition to the sugars already noted. The concentrations of sugars in mature whiskies (of the order of 100 mg/L) are too low to suggest any sweetening effect on the beverage. Phenols are also detectable in mature whisky, although some of these probably arise during mashing (Steinke and Paulson, 1964) or from malt produced using peat-fired kilns (MacFarlane, 1968). However, Salo *et al.* (1976) reported an increase during a one year maturation of a Scotch malt whisky in the concentration of eugenol, which is a major phenol extracted from oak chips by ethanol (Suomalainen and Lehtonen, 1976). Also present in mature whiskies are sterols, which may precipitate in bottled whisky stored at room temperature. Black and Andreasen (1973) found campesterol, stigmasterol and sitosterol in mature bourbon, in addition to sitosterol-D-glucoside, although the possibility that some of these were formed during mashing cannot be excluded. Finally, reference has already been made to the whisky lactone, ß-methyl-octalactone, and its origin.

Not surprisingly, the nature and amounts of compounds extracted from charred oak wood depend on the ethanol concentration of the whisky. It is to some extent an advantage to mature whisky at a high proof, since this requires fewer barrels and saves on storage space. Until 1962 the US Treasury Department limited the barrelling proof of whisky to a maximum of 110° US proof (55° GL). In anticipation of this limit being raised to 125° US proof (62.5° GL), Baldwin and Andreasen initiated a series of experiments in 1962 to establish the importance of barrelling proof on changes in color and concentrations of organoleptically-important compounds during maturation of bourbon whiskies. Their report in 1973 indicated that color intensity and congener concentration of whiskies matured for 12 years decreased as the barrelling proof was raised from 109° US proof (54.5° GL) to 155° US proof (77.5° GL). The one exception was the higher alcohol content, which remained approximately constant.

Is it really Scotch?

Analysis of the variety of compounds in spirit is also of interest for reasons of identifying or authenticating product origins or types. Adam *et al.* (2002) investigated whether malt, blended and grain whiskies could be differentiated based on content of various metals. While it was not possible to define a 'metal fingerprint' that would identify a whisky as to origin, malt whiskies had markedly higher concentrations of copper than blended or pure grain whiskies. Differences were significant with malt whiskies containing 385-480 ng Cu/mL and grain and blended whiskies contained 131-242 ng/mL. Since malt Scotch is produced in traditional copper pot stills while grain whisky used in blending is made in continuous patent stills, copper content was suggested as a means of distinguishing a malt Scotch from a blended product.

Another approach to distinguishing between malt and blended products is based on the differences between barley, which is used to produce malt Scotch and the corn (maize) that is typically used in production of grain whisky. Parker *et al.* (1998) found that the ratio of ^{12}C and ^{13}C isotopes in volatiles such as acetaldehyde, ethyl acetate, n-propanol and others differed among whiskies. This was a reflection of the different types of photosynthetic metabolism of barley, a cool season plant with C3 metabolism, versus maize, which being adapted to hotter climates has C4 metabolism.

There is no doubt value in being able to chemically define the special characteristics that distinguish an ordinary whisky from malt Scotch. Authentication is often needed; and a thorough understanding of the chemical basis of organoleptic properties might pave the way to production of even finer whiskies than those we enjoy most today. For the moment, however, the magic remains. The distiller's art continues to elude the chemist.

Acknowledgments

The author wishes to thank his friends and colleagues in whisky production in many countries of the world for their cooperation and willingness to supply information on the processes of whisky production. Particular thanks are due to Dr. Gareth Bryan-Jones of United Distillers, Menstrie, Scotland, for his assistance in providing research reports.

References

Anderson, R.J., G.A. Howard and J.S. Hough. 1972. 'Proceedings of the European Brewing Convention, Vienna. p 253.

Aylott, R.I., A.H. Clyne, A.P. Fox and D.A. Walker. 1994. Analyst 119:1741-1746.

Baldwin, S. and A.A. Andreasen. 1973. Journal of the Association of Official Analytical Chemists 57:940.

Baldwin, S., R.A. Black, A.A. Andreasen and S.L. Adams. 1967. Journal of Agriculture and Food Chemistry 15:381.

Barbour, E.A. 1983. Taxonomy of some lactic acid bacteria from scotch whisky distilleries. PhD Thesis, Heriot-Watt University.

Barnard, A. 1887. The whisky distilleries of the United Kingdom. Proprietors of Harpers Weekly Gazette, London, pp. 457.

de Becze, G.I. 1967. Encyclopedia of Industrial Analysis. Vol. 4:462. Interscience Publications, New York.

Beech, F.W. 1972. Progress in Industrial Microbiology I I:178.

Black, R.A. and A.A. Andreasen. 1973. Journal of the Association of Official Analytical Chemists 56:1357.

Black, R.A. and A.A. Andreasen. 1974. Journal of the Association of Official Analytical Chemists 57:111.

Black, R.A., A.A. Rosen. and S.L. Adams. 1953. Journal of the American Chemical Society 75:5344.

Bober, A. and S.W. Haddaway. 1963. Journal of Gas Chromatography 1 12: 8.

Brander, M. 1975. A Guide to Scotch Whisky. Johnson and Bacon, Edinburgh. p. 96.

Bryan-Jones, G. 1976. Lactic Acid Bacteria in Beverages and Foods. Proceedings of the Fourth Long Ashton Symposium. (J.G. Carr, C.V. Cutting and G.C. Whiting, eds). Academic Press, London. pp. 165-175.

Cameron Taylor, I.B. 1970. Whisky Export, 2nd issue, June-Sept. Aldiffe Publishing Co. Ltd., Wilmslow, Cheshire.

Carter-Tijmstra, J. 1986. Proceedings of Second Aviemore Conference on Malting, Brewing and Distilling. pp. 413-416.

Connor, J.M., A. Paterson and J.R. Piggot. 1990. Proceedings of Third Aveimore Conference on Malting, Brewng and Distilling. pp. 460-463.

Cook, R. 1990. Proceedings of Third Aveimore Conference on Malting, Brewing and Distilling. pp. 237-243.

Court, R.E. and V.H. Bowers. 1970. Process Biochemistry 5:17.

Cromwell, E.A. and J.F. Guymon. 1963. American Journal of Enology and Viniculture 12:214.

Daiches, D. 1969. Scotch whisky, its past and present. Andre Deutsch, London. p. 168.

Dolan, T.C.S. 1976. Journal of the Institute of Brewing. 82:177.

Duncan, R.E.B. and J.M. Philp. 1966. Journal of the Science of Food and Agriculture 17:208.

Dunnett, A. 1953. The land of scotch. Scotch Whisky Association, Edinburgh. p. 170.

Geddes, P.A. and H.L. Riffkin. 1989. Distilled Beverage Flavour (ed. Piggot, J.R. Ellis, Horwood, London. pp. 193-199.

Griffin, O.T. 1972. Process Biochemistry 7:17.

Gunn, N.M. 1935. Whisky and Scotland. Routledge, London. p 198.

Harrison, G.A.F. 1970. Journal of the Institute of Brewing 76:486.

Harrison J.S. and J.C.J. Graham. 1970. In: *The yeasts*. (A.H. Rose and J.S. Harrison, eds.). Vol. 3:283-348. Academic Press, London.

Heukelekian, H. and A. Heller. 1940. Journal of Bacteriology 40:547.

Hopkins, R.H. and S. Wiener. 1955. Journal of the Institute of Brewing 61:493.

Hough, J.S., D.F. Briggs and R. Stevens. 1971. Malting and brewing science. Chapman and Hall Ltd., London. p. 678.

Institute of Brewing Analysis Committee, Recommended methods of analysis. 1975. Journal of the Institute of Brewing 81:368.

Jacobs, M.B. 1947. American Perfumery and Essential Oil Review. p 157.

Jack, F. 2003. Development of guidelines for the preparation and handling of sensory samples in the Scotch whisky industry. J. Inst. Brewing 109:114-119.

Kahn, J.H. 1969. Journal of the Association of Official Analytical Chemists 52: 1166.

Kahn, J.H., P.A. Shipley, E.G. Laroe and H.A. Conner. 1969. Journal of Food Science 34:587.

Kleber, von W. and N. Hurns. 1972. Brauwissenschaft 25: 98.

Kulka, D. 1953. Journal of the Institute of Brewing 59: 285.

Liebmann, A.J. and B. Scherl. 1949. Industrial and Engineering Chemist 41:534.

Lloyd Hind, H. 1948. Brewing Science and Practice, Vol. 1:505. Chapman and Hall, London.

Lyons, T.P. 1974. The Brewer. p 634.

McDowell, R.J.S. 1975. The Whiskies of Scotland. 3rd edit. John and Murray, London. p. 166.

MacFarlane, C. 1968. Journal of the Institute of Brewing 74: 272.

MacFarlane, C., J.B. Lec and M.B Evans. 1973. Journal of the Institute of Brewing 79:202.

McGuire, E.B. 1973. Irish Whiskey. Gill and MacMillan, Dublin. p. 462.

MacKenzie, K.G. and M.C. Kenny. 1965. Journal of the Institute of Brewing 71:160.

Mann, E.A. 1911. Government Analyst for Western Australia. Perth, W. Australia. pp. 1-12.

Merritt, N.R. 1967. Journal of the Institute of Brewing 73:484.

Morrison, R.L. 1962. American Journal of Enology and Viniculture 13:159.

Murtagh, J.E. 1970. M.Sc. Thesis: University College of North Wales, Bangor.

Nettleton, J.A. 1913. The manufacture of whisky and plain spirit. Cornwall and Sons, Aberdeen. p. 616.

Nishimura, K. and M. Masuda. 1971. Journal of Food Science 36:819.

Nishimura, K and R. Matsuyama. 1989. The Science and Technology of Whiskies. (J.R. Piggott, R. Sharp and R.E.B. Duncan, eds.). Longman Scientific and Technical, Harlow. pp. 235-263.

Otsuka, K. and K. Morinaga and S. Imai. 1963. Nippon Jozo Kyokai Zasshi 59:448.

Parker, I.G., S.D. Kelly, M. Sharman, M.J. Dennis and D. Howie. 1998. Investigations into the use of carbon isotope ratios (13C/12C) of Scotch whisky congeners to establish brand authenticity using gas chromatography - combustion isotope ratio mass spectrometry. Food Chem. 63:423-428.

Perry, D.R 1986 Proceedings of Second Aviemore Conference on Malting, Brewing and Distilling. pp. 409-412.

Pert, D. and E. Woolfendon. 1986. Proceedings of Second Aviemore Conference on Malting, Brewing and Distilling. pp. 417-424.

Philip, J.M. 1989. The Science and Technology of Whiskies. (J.R. Piggott, R. Sharp and R.E.B. Duncan, eds). Longman Scientific and Technical, Harlow. pp. 264-294.

Preece, I.A. 1947. Journal of the Institute of Brewing 53:154.

Preece, I.A. 1948. Journal of the Institute of Brewing 54:141.

Preece, I.A. and M. Shadaksharaswamy. 1949a. Journal of the Institute of Brewing 55:298.

Preece, I.A. and M. Shadaksharaswamy. 1949b. Biochemical Journal 44:270.

Preece, I.A. and M. Shadaksharaswamy. 1949c. Journal of the Institute of Brewing 55:373.

Pyke, M. 1965. Journal of the Institute of Brewing 71:209.

Rae, I.L. 1967. Process Biochemistry I, 8:407.

Ritter, G.J. and L.C. Fleck. 1923. Industrial and Engineering Chemistry 15:1055.

Robb, J.M. 1950. Scotch Whisky. W. and R. Cham-bers, London. p 197.

Ross, J. 1970. Whisky. Routledge and Kegan Paul, London. p. 158.

Royal Commission on Whisky and Other Potable Spirits. 1909. Appendix Q, p. 229.

Salo, P. 1970. Journal of Food Science 35:95.

Salo, P. 1975. Proceedings of the International Symposium on Aroma Research. Central Institute for Nutrition and Food Research, TNO, Zeist, Netherlands. p 121.

Salo, P., L. Nykänen and H. Suomalainen. 1972. Journal of Food Science 37:394.

Salo, P., M. Lehtonen and H. Suomalainen. 1976. Proceedings of the Fourth Symposium on Sensory Properties of Foods. Skövde, Sweden.

Schidrowitz, P. 1902. Journal of the Chemical Society. p 814.

Schidrowitz, P. and F. Kaye. 1905. Journal of the Chemical Society. p. 585.

Scotch Whisky Order. 1990. Stationery Office, London.

Sillett, S.W. 1965. Illicit Scotch. Beaver Books, Aberdeen. p. 121.

Simpson, A.C. 1968. Process Biochemistry 3, 1:9.

Simpson, B., A. Troon, S.T. Grant, H. MacDiarmid, D. MacKinlay, J. House and R. Fitzgibbon. 1974. Scotch Whisky. Mac-Millan, London. p. 120.

Singer, D.D. and J.W. Stiles. 1965. Analyst 90:290.

Stark, W.H. 1954. Industrial Fermentations (L.A. Underkofler and R.J. Hickey, eds). Chemical Publishing Company, New York. Vol. 1, pp. 17-72.

Steinke, R.D. and M.C. Paulson. 1964. Journal of Agricultural and Food Chemistry 12: 381.

Suomalainen, H. 1971. Journal of the Institute of Brewing 77:164.

Suomalainen, H. and M. Lehtonen 1976. Kemia-Kemi 3, 2:69.

Suomalainen, H. and L. Nykänen. 1966. Journal of the Institute of Brewing 72:469.

Suomalainen, H. and L. Nykänen. 1970a. Naeringsmidelidustrien 23:15.

Suomalainen, H. and L. Nykänen. 1970b. Process Biochemistry. July 1.

The Scotch Whisky Order. 1990. Statutory Instruments No 998. HM Stationery Office, London.

Thorne, C.B., R.L. Emerson, W.J. Olsen and W.H. Paterson. 1945. Industrial and Engineering Chemistry 37:1142.

Valaer, P. 1940. Industrial and Engineering Chemistry 32:935.

Valaer, P. and W.H. Frazier. 1936. Industrial and Engineering Chemistry 28:92.

Walker, J.W., D.M. Vaughan and H.L. Riffkin. 1990. Proceedings of Third Aveimore Conference on Malting, Brewing and Distilling. pp. 421-426.

Warricker, L.A. 1960. Journal of the Science of Food and Agriculture 11:709.

Wilson, J. 1973. Scotland's malt whiskies, a dram by dram guide. Famedram Ltd., Gartochan, Dunbartonshire, Scotland. p. 109.

Wilson, J. 1975. Scotland's distilleries. a visitor's guide. Famedram Ltd., Gartochan, Dunbartonshire, Scotland. p. 104.

Wilson, Ross. 1959. Scotch made easy. Hutchinson, Ltd., London. p 336.

Wilson, Ross. 1970. Scotch: the formative years. Constable, London. p 502.

Wilson, Ross. 1973. Scotch, its history and romance. David and Charles, Newton Abbot, Devon, England. p 184.

Wobben, H. J., R. Timmer, T. ter Heide and P. de Valois, P. 1971. Journal of Food Science 35:464.

Zobell, C.E. 1943. Journal of Bacteriology 46:39.

Chapter 15

Tequila production from agave: historical influences and contemporary processes

MIGUEL CEDEÑO CRUZ

Tequila Herradura, S.A. de C.V. Ex-Hda San Jose del Refugio Amatitán, Jalisco, México

Introduction: agave beverage alcohol products

Tequila is classically associated with Mexico and has been long identified as more than just the national drink, but a symbol of our culture and environment. As the reach of the global trade has extended, tequila is now consumed around the world.

This alcoholic beverage is obtained from the distillation of fermented juice of the agave plant, *Agave tequilana* Weber var. azul. *A. tequilana* is often confused with various cacti growing in same desert environment, but they belong to different botanical families: agaves to *Agavaceae* and cacti to *Cactaceae* families, respectively.

There are two classifications of tequila based on agave content: 100% tequila, produced using only agave, and 51% tequila, which contains sugars from other sources such as cane and corn added in the fermentation step, at up to 49% by weight. It is important to note that only tequila 51% can be exported in bulk outside Mexico since 100% tequila must be state-bottled.

Based on maturation, there are four classifications of tequila: silver, without maturation, gold, containing permitted additives and colors (generally caramel color), aged tequila (Reposado) matured at least two months in white oak tanks with a maximum capacity of 159 gallons or barrels, and extra-aged (Añejo) matured at least 12 months in white oak barrels.

More than 300 brands (labels) of tequila are produced in the 104 distilleries registered in Mexico by the Tequila Regulatory Council (CRT by its acronym from the Spanish: Consejo Regulador del Tequila), which verifies compliance with the Mexican official standard (Secofi, 1994) through routine on-site inspections.

Tequila is differentiated from the beverage known as 'mezcal' by the type of agave used in its production. Mezcal is made from *Agave potatorum,* which grows in the states of Oaxaca, San Luis Potosi, Guerrero and Zacatecas. Most mezcal producers use a rudimentary fermentation and distillation process (Sánchez, 1991). There is no technical reason for, or any improvement in the organoleptic characteristics from, the worm inside the bottles of some mezcal brands. The worm is primarily a commercial ploy. Worms are grown in agave plants and introduced manually in the bottling line.

'Pulque' is another beverage obtained by fermentation of the juice obtained from several species of agave, *A. atrovirens* and *A. salmiana* among others, by a complex succession of yeast and bacteria that produce ethanol, a diversity of chemical compounds, and some polymers that give a sticky consistency to the final product (Rzedowski, 1978; Sánchez Marroquin and Hope, 1953*)*. Pulque is sometimes mixed with fruits or vegetables, but has poor stability as it is neither distilled nor pasteurized.

Agave plants still serve as food in some states of Mexico; and other fermented regional beverages are produced (e.g., 'Sotol' in the state of Chihuahua and 'Bacanora' in the state of Sonora), but only tequila and more recently mezcal have reached international recognition. Another difference between tequila and mezcal or other regional drinks is that both are subject to an official standard that for tequila is NOM-006-SCFI-1994 (Secofi, 1994), and production is supervised by the Mexican government. Table 1 shows the main differences among the regional beverages.

Origin and history of tequila

The word 'tequila' is believed to originate from the tribe of *ticuilas* who long ago inhabited the hillside of a volcano bearing the same name located near the city of Tequila. Another possible origin is the Nahuatl word *tequitl,* which means *work* or *employment*, and the word *tlan,* which means *place*. Therefore 'tequila' would mean *place in which labor or work is done*.

The oldest reference to the existence of agave and its different uses is from the era before the Spaniards in several codices preserved to the present time (a codex, from the Latin *codex* meaning board or writing tablet, is a manuscript volume, especially of a classic work or scripture). The most important is the *Tonalmatlnahuatl* codex, which notes that certain tribes had learned to cook agave plants and used them as food and to compensate for the lack of water in desert lands. Also, these tribes discovered that cooked agave when soaked in water would ferment, producing a much appreciated beverage. In fact, this primitive and rudimentary method was used for centuries to produce beverages from agave, considered a sacred plant possessing divine properties. In other codices, such as *Nutall, Laud, Borgia* and *Florentine,* there are many references to uses of the agave plant for soap manufacture, a source of fiber, footwear, medicine and sewing needles as well as thread, paper and rope. In fact, Indians distinguished the different species of agave by color, size, stem, leaf width and the different uses given each plant (Muria, 1990). The great religious importance of agave was apparent in those codices, as only warriors and priests used fermented drinks in ritual ceremonies.

In pre-hispanic Mexico the general name for all species of agave (or mezcal as it is also known) was *Metl,* which is a representation of the goddess Mayahuel (Figure 1). The alcoholic drink produced was called *Iztac octli* (white wine). The first Spaniards to arrive in Mexico referred to the plant as 'maguey', a name used for an identical plant they had seen in the Caribbean Islands, where they first encountered new world plants and animal life (Bottorff, 1971). It was not until the arrival of the Spaniards, who brought knowledge of distillation techniques, that tequila took its present form (Goncalves, 1956). Tequila is considered the first distilled beverage in North America and has been given several different names including agave wine, mezcal wine and finally tequila.

Table 1. Agave alcoholic beverages produced in Mexico.

Beverage	Agave species	Distillation process	Producing region	Fermentation: main microorganisms
Tequila	*tequilana*	Yes	Jalisco	Yeast
Mezcal	*potatorum, salmiana, angustifolia*	Yes	Oaxaca, San Luis Potosi	Yeast
Bacanora	*pacifica, palmieri, angustifolia*	Yes	Sonora	Yeast
Sotol	*angustifolia, dasylirion*	Yes	Chihuahua	Yeast
Pulque	*atrovirens, salmiana*	No	Central	Yeast, bacteria

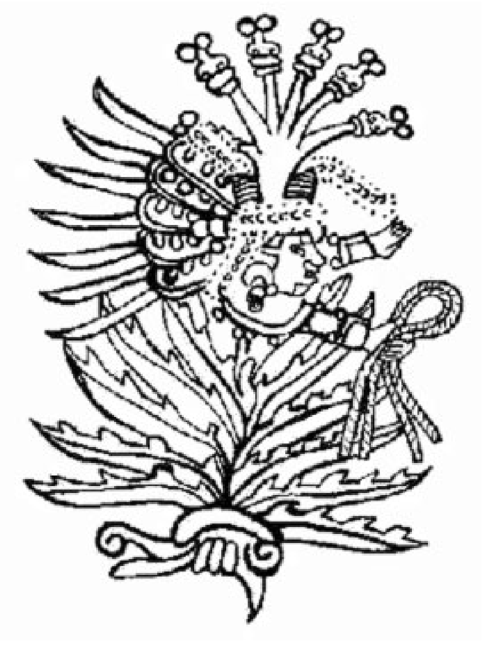


Figure 1. Representation of the goddess Mayahuel.

There are specific regions in Mexico where agave should be grown, where tequila must be produced and where 100% tequila must be state-bottled. This area comprises the municipalities of the following states: Jalisco (all), Nayarit (8), Michoacan (30), Guanajuato (7) and Tamaulipas (11) (Figure 2). The oldest, the Tequila-Amatitán region that comprises the Amatitán village, developed at the end of the 17th century. The second region, the Jalisco Highlands, appeared in the last decade of the 19th century (Luna, 1991).

The first tequila production process with a commercial purpose was established in the city of Tequila around the end of the 18th century. The main consumers were in the mining zones located in the state of Jalisco. The Spaniards tried to suppress consumption of tequila in order to reduce competition for brandy and other wines imported from Spain with a decree signed by Carlos III forbidding sale and production of tequila under the pretext that its consumption was the cause of several illnesses. The results were negative. The governor of the region later issued a decree imposing a tax on tequila in order to enrich the royal coffers, thus permitting its sale in all of New Spain. By the end of the 19th century, expansion of the tequila industry, helped by the railway, was evident. However it was not until the first casks were exported to the US that tequila was known beyond Mexico's borders.

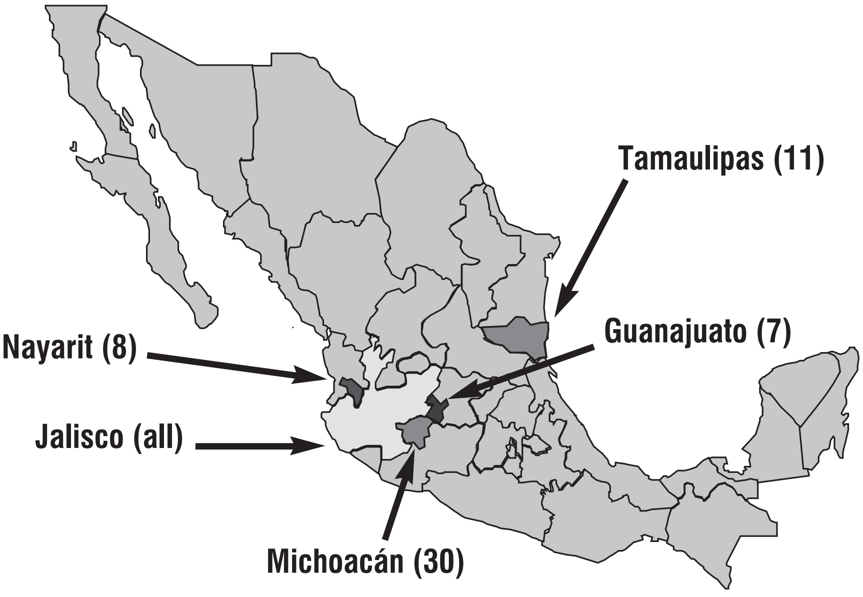


Figure 2. States and municipalities for agave cultivation and tequila production in Mexico.

The agave plant

According to Granados (1985), the genus *Agave,* which means 'noble' in Greek, was defined by Linneaus in 1753 when he described the plant *A. americana* as the first agave species known to science. Agave plants, which are often confused with cacti, belong to the family Agavaceae and are succulent plants with spirally-arranged leaves forming a rosette. Some have definite trunks, but more often they are nearly stemless. The leaves are bluish green in color, over 1 m long in mature plants, and end in a sharpened brown thorn. As Backman (1944) pointed out, the widespread distribution of some 300 species, combined with the fact that the plants require approximately 8-12 years to mature and hybridize very easily, make the taxonomic and phylogenetic study of the genus *Agave* extremely complicated and very difficult. The family Agavaceae includes 20 genera and nearly 300 species. Of these, around 200 are found in Mexico. In Figure 3, a diagram of an agave plant in soil shows the main structural features.

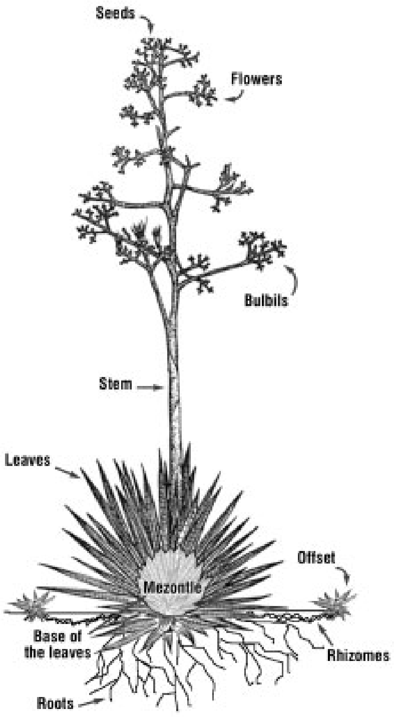


Figure 3. Structure of *Agave tequilana* Weber var. azul.

Agave has commercial importance as a fiber source; and this industry developed in the 19th century in Mexico mainly in the Yucatan state with the cultivation of henequen, *A. fourcroydes*, to export fiber to Europe. However, the Philippines, Indonesia and Tanzania in Africa competed in this market with fiber produced by *A. sisalana.* The use of agave as a forage for cattle is a recent development for milk production, but must be enriched with a protein source. Other agaves such as *A. americana, A. victoria regina, A. filifera* and *A. stricta* among others are used as ornamentals.

In the beverage industry, *A. salmiana* and *A. atrovirens* are valued for pulque production, *A. potatorum, A. salmiana* and *A. angustifolia* for mezcal production, *A. pacifica, A. palmieri* and *A. angustifolia* for the elaboration of bacanora and *A. dasylirion* and *A. angustifolia* for sotol production. Finally, *A. tequilana* Weber var. azul, named around 1900 by the German botanist Weber (Diguet, 1902), is used to produce tequila.

CULTIVATION AND HARVEST

The blue agave, as it is known, is the only species out of hundreds of Agavaceae with the appropriate characteristics for tequila production. These include a high inulin concentration, low fiber content and the chemical compounds present in the plant that contribute to the final taste and flavor of tequila to give the beverage its particular character. Some have attempted to produce tequila in other states with other agave species, but without success. The blue agave is mainly cultivated in the state of Jalisco in the two regions with the right climate and soil composition for its growth, namely the Highlands and the Tequila-Amatitán region. The temperature conditions for good agave yields are a minimum of 3°C, an optimum of 26°C and maximum of 47°C. Soil should be fertile but not very deep, 30 to 40 cm. Good drainage is required to avoid effects of flooding, which are very harmful for development of the agave. Agave are planted at 800 to 2500 m above sea level where the annual rainfall is about 800 to 900 mm. The correct planting time is immediately before the rainy season, from June to September, so that the plants do not suffer from water stress during the first year of growth.

Propagation is accomplished by vegetative means in agave. Sexual reproduction via seeds is not usual. Asexual bulbils develop in the inflorescence at the base of the flowers, producing small plants that after some time detach themselves from the floral peduncle and fall to the soil where they root. Another mode of asexual propagation is by suckers, which are a characteristic type of lateral bud or branch developing at the base of the main stem. Plants developing near each mother plant are separated at the age of 3-4 years. These baby plants are called 'first-class seed' because they are better and healthier (Sánchez, 1991). In practice, people use the word 'seed' to refer to such young plants, but from a botanical point of view these are rhizome shoots or suckers (Valenzuela, 1992). Plant cell culture is used by some tequila companies in order to maintain plants of consistent quality, reduce the cost of labor for sowing the plants, eliminate use of fungicides and antibiotics during sowing, and have bigger plants with higher inulin content at the end of the cultivation period.

Land for agave cultivation must be cleared and deep-ploughed, sometimes twice. Agave is planted approximately 2-4 m apart in straight lines called ruts. Sowing is done by hand in holes 15 cm in depth. Plant density is around 2000-4000 plants per hectare, depending on the plantation system used; and yields can be between 30,000 and 200,000 kg/ha, assuming that the weight of a harvested plant varies from 15 to 50 kg. This variation is caused by differences in soil conditions, quality of plants sown, rainfall, pests and fertilization. Sometimes agave is interseeded with nitrogen-fixing crops such as peanuts, beans, chickpeas or soybeans. After the agave has been in the soil for a year, visual inspection is conducted and sick or dead plants are replaced. This operation is called re-seeding. The percentage of dead plants depends on soil and plant characteristics, but is generally from 8 to 15% (Pérez, 1990).

Agave plants regularly host borer insects that live in the stems, leaves and fruits during the larval stage. These include butterflies of the family Megathymidae and moths of the family Prodoxidae. Also, the fungi *Diplodia theobromae* and *Colletotrichum agavae* may cause serious damage to agave leaves (Halffter, 1975; Agricultural Research Service, 1972).

Agave plants, adapted to hot and very arid conditions, employ crassulacean acid metabolism (CAM). Crassulacean acid metabolism is similar to photosynthetic processes in other hot temperature-adapted plants in that the enzyme phosphoenolpyruvate (PEP) carboxylase is used to fix CO_2 into a 4-carbon compound (C_4 metabolism). However in most hot season plants the light-dependent reactions and CO_2 fixation are separated in different cells of the leaf. In contrast, in plants with crassulacean acid metabolism such as cacti and agave these reactions occur at different times of the day. Carbon dioxide (CO_2) enters the leaf through stomata with the simultaneous loss of water. By only requiring the stomata to be open during the cooler night for CO_2 uptake and keeping them closed during the hotter day, the agave plants lose much less water (Ehrler, 1967). Malic acid formed by CO_2 fixation using PEP carboxylase accumulates during the night, and is broken down during the day (Figure 4). The CO_2 that is released can be re-fixed by the normal photosynthetic Calvin cycle (C_3 metabolism) to produce sugars and then inulin.

The thick cuticle overlying the epidermis, which is quite evident in tequila agave, apparently prevents damage to the leaf from high temperatures (Casado and Heredia, 2001). This waxy cuticle produces turbidity in tequila because it dissolves in the distillation step and produces a haze in the final product when it is diluted or cooled. One way to avoid this is to treat tequila with activated charcoal and to filter it through pure cellulose filter pads. This, unfortunately, results in the loss of some aromas.

Agave fertilization is determined by soil composition, plant age, and the type of chemical compound used. The normal procedure is to use urea as a nitrogen source in amounts of 30-70 g per plant incorporated directly into the soil. In some areas, phosphorus and potassium fertilization is also required. As some agave regions are also involved in cattle, swine, and chicken production, manure is sometimes employed for fertilization (GEA, 1992).

The average maturity time for agave is seven years; and at this time the content of inulin is at its highest level (Mendez, 1999). Every year

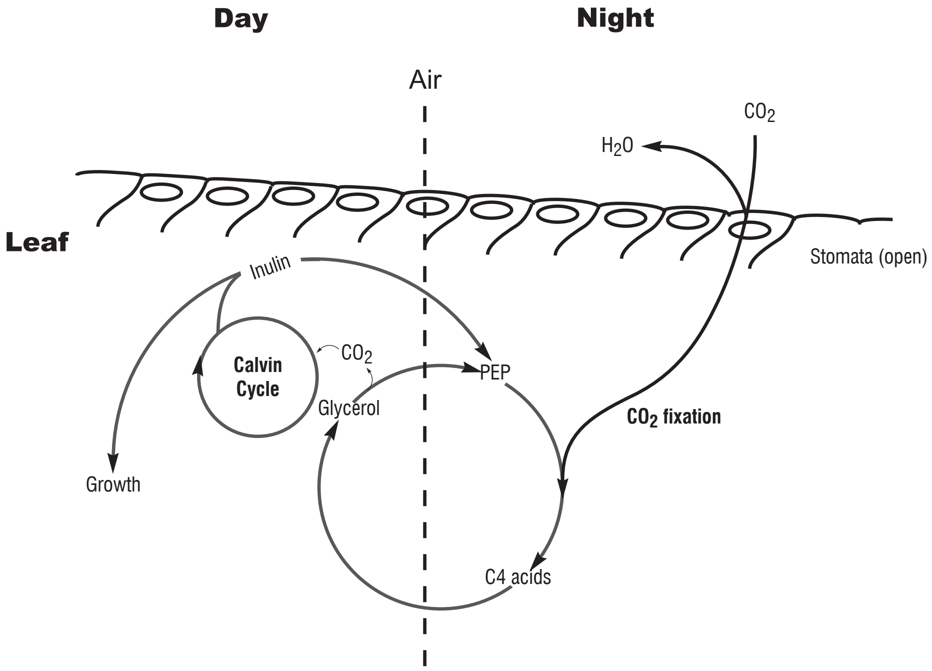


Figure 4. Schematic representation of carbon dioxide fixation by agave (PEP: Phosphoenolpyruvate).

following planting, fields are cultivated to loosen the soil and weed and pest control is carried out. Each plant matures individually. Harvest begins in the seventh year. The leaves are cut from the base and left in the field to recycle nutrients. The harvested plants free of leaves look like large pineapples and weigh from 20 to 90 kg. They are transported to the distillery. Only the better plants, meaning those of good size and high inulin content (measured as reducing sugars), are harvested; but on the 12th year, all plants remaining (the weakest plants) are cut. These are called ‘drag’ and are generally discarded. Agave composition varies seasonally, but an average would be (wet basis) 27% (w/w) reducing sugars, a juice content of 0.572 ml/g and a pH of 5.2.

In Figure 5 the amount of agave harvested for tequila production is presented for the period 1995-2002. These figures show, first of all, a

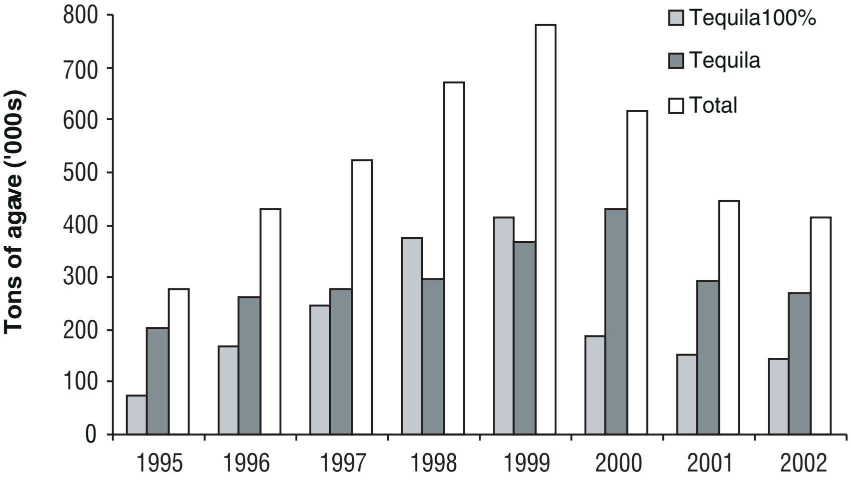


Figure 5. Agave harvested for tequila production (CRT, 2002).

decrease in total agave production. This reflects the shortage caused by disease in agave plantations in the year 2000, which was mainly due to a bacteria *Erwinia carotovora* and a fungus *Fusarium oxysporum,* which destroyed more than 25% of the total agave plants for tequila. From this graph, it is evident that the agave utilization for 100% tequila is growing, since that beverage is being recognized as a premium product not only in Mexico but also overseas.

Tequila production

Tequilas differ greatly depending on agave quality and origin (Highlands or Tequila-Amatitán regions). The production process also strongly influences quality of the final product. Some distilleries still employ rudimentary production methods, just as they did several decades ago (Pla and Tapia, 1990). Most companies, however, employ technological advances that improve process efficiency and consistency; and some have implemented a ISO-9000 standard. Whatever the process used, tequila manufacture comprises four main steps: cooking, milling, fermentation and distillation. An extra step, maturation, is required for aged products. Figure 6 shows a flow diagram for the tequila production process, which is explained in detail in the following paragraphs.

Processing harvested agave

Agave is transported from the fields to the factories as soon as possible to avoid weight losses, because today most distilleries pay by weight and not by inulin content. The heads are unloaded from the truck in the receiving area of the factory and must be protected from the sun and rain in order to avoid withering and fungal growth. Although the agave has already been inspected during growth and at harvest, it is examined again to reject visually unacceptable

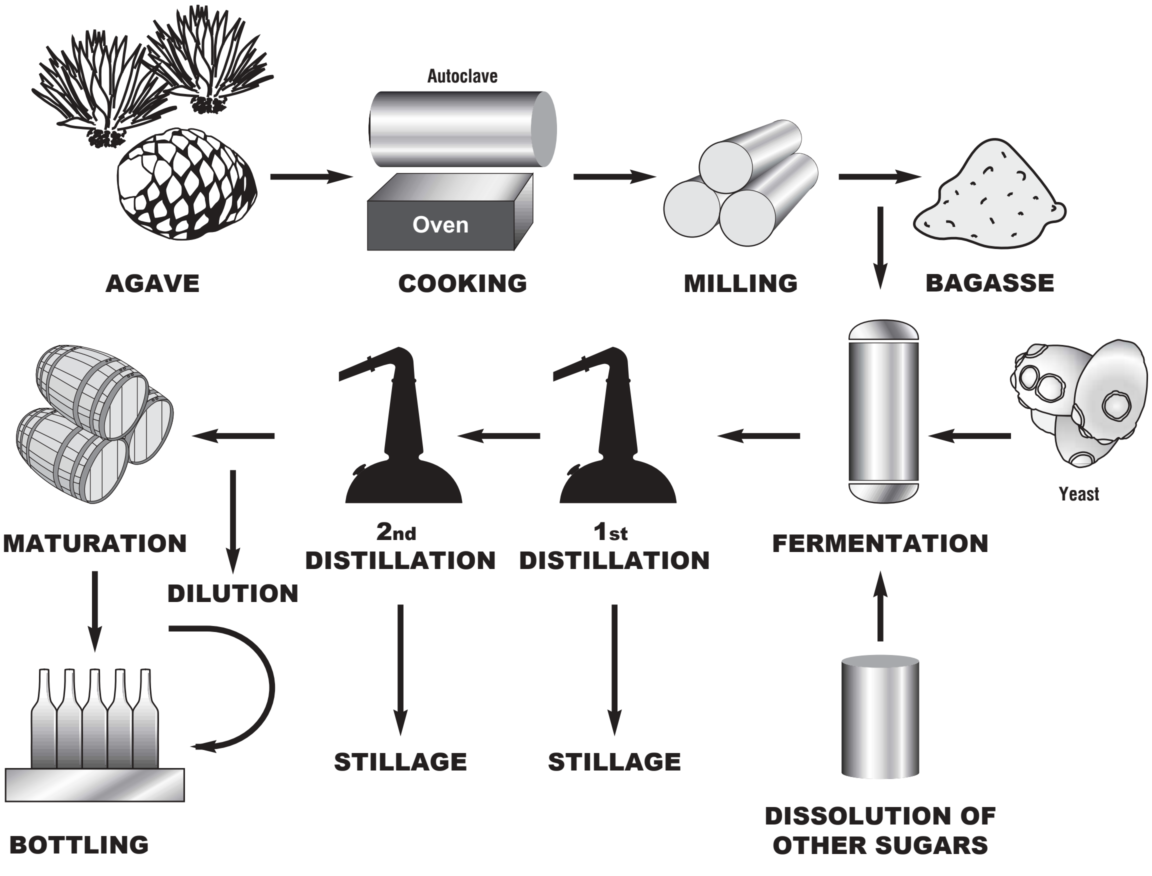


Figure 6. Flow diagram for the tequila production process.

plants or those damaged by pests. At this point, a representative sample of agave is taken for laboratory analysis. Modified AOAC (1990) procedures are used to determine reducing sugar content (after acid hydrolysis of inulin) along with pH, moisture, dry weight, juice and ash content.

Agave heads usually weigh between 20 and 60 kg, although some can reach 100 kg. They are cut to sizes that facilitate uniform cooking and handling. Different agave cutting systems exist, but the use of axes and a specialized tool called 'coa' are the most popular. The heads are cut in halves or quarters, depending on the weight, and the pieces are arranged manually in an oven or autoclave. Band saws can also be used to cut the agave heads, since they are faster and less labor-intensive than the manual procedure, but the belts break frequently because of the resinous consistency of agave. Some factories tear uncooked agave first with a knife and place the resulting pieces mixed with water into autoclaves to be cooked.

OTHER RAW MATERIALS FOR TEQUILA PRODUCTION

When producing 100% agave tequila, the only source of carbohydrate is the inulin hydrolyzed from agave in the cooking step. Inulin, a polymer of fructose with a terminal glucose, belongs to the family of fructans (Figure 7). After starch, fructans are the most abundant non-structural polysaccharide found in nature.

For other kinds of tequila the law permits use of other sugars in amounts up to 49% by weight in wort formulation. There are no legal specifications regarding the type of adjunct sugar sources to be used in tequila manufacture; and theoretically any kind of fermentable sugar is allowed in wort formulation. In practice and from an economic point of view, only cane sugar, Piloncillo, cane molasses and acid- or enzyme-hydrolyzed corn syrup are employed. Cane sugar is received in 50 kg bags and stored in a dry, cool place for subsequent utilization. Piloncillo consists of brown cones of crystallized complete cane juice, sometimes individually wrapped in corn or cane leaves and packed in sacks. Cane molasses is also used but it is difficult to handle and there is risk of spoilage over a long storage period. Acid- or enzyme-hydrolyzed corn syrup may be used to formulate the wort. All sugars used in wort formulation are routinely analyzed by measuring solids content and reducing and fermentable sugar content.

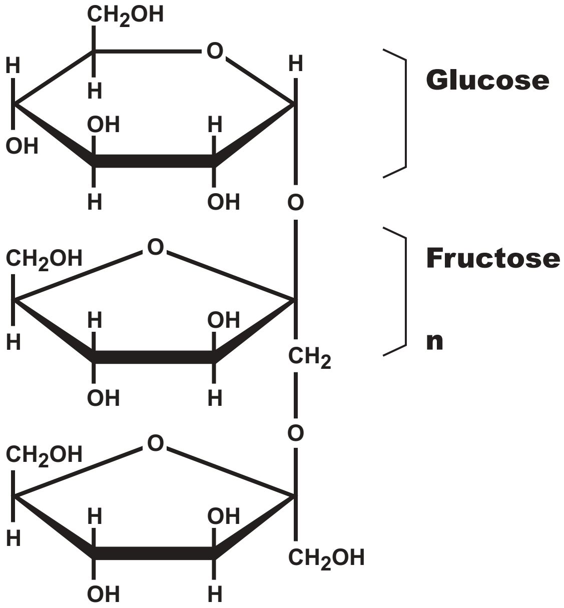


Figure 7. Structure of inulin, in which chain length, n, varies from 3 to 19, depending on the maturity of the agave plant (Mendez, 1999).

The cooking step: hydrolysis of inulin

Cooking the agave serves three purposes. First, the low pH (4.5) together with the high temperature hydrolyzes inulin and other components of the plant. A recent study (Pinal, 2001) showed that the concentrations of furfural, 5-hydroxymethyl furfural, 5-methylfurfural and 2-acetylfuran increase with length of cooking time. These components are important as they may influence fermentation rate as well as the organoleptic characteristics of the final tequila. For this reason, time control during cooking is very important. In addition, cooked agave has a soft consistency that facilitates the milling operation.

In the pre-Hispanic era, agave was cooked in holes filled with stones heated using wood for fuel. The stones retained the heat for the time needed to cook the agave. Nowadays some distillers have replaced the heated stone holes with brick ovens and heating is accomplished by steam injection after the chopped raw agave has been introduced into the oven. Oven cooking is slow, and steam injection lasts around 36-48 hrs to obtain temperatures of 100°C. After that period, the steam is shut off and the agave is left in the oven for a further two days to complete the cooking process. During this step, a sweet liquid called 'cooking honey' is collected and used later as a source of free sugars, mainly fructose. Also during this step some of the sugars are caramelized; and some of the compounds that contribute significantly to the aroma and flavor in wort formulation are due to its high content of fermentable sugars (>10% w/v). Finally, the oven door is opened to allow the cooked agave to cool. The agave is then ready for milling.

In most distilleries brick ovens have been replaced by steel autoclaves. Autoclaves have superior efficiency and allow good pressure and temperature control, enabling homogeneous and economic cooking. In a typical autoclave cooking operation, steam is injected for 1 hr so that steam washes the agave. This condensed liquid is called 'bitter honey' and is discarded because it contains waxes from the agave cuticle and has a low sugar content (<1 % w/w). Steam is injected for an additional 6 hrs to obtain a pressure of 1.2 kg/cm^2 and a temperature of 121°C. At the end of that time the agave remains in the autoclave for another 6 hrs without additional steam, cooking slowly in the remaining heat. This step produces a syrup with a high sugar concentration (>10% by weight) that is later used to formulate the initial wort. To calculate the yield and efficiency of this step, the amount of cooking honey and its reducing sugar content as well as the cooked agave are measured. Figure 8 illustrates the temperature profile over time for cooking agave in an autoclave compared with an oven.

The main difference between autoclaved and oven-baked agave is that careful control of cooking time, temperature and steam pressure must be maintained in autoclaves to prevent overcooking or burning the agave. Overcooking gives a smoky taste to the tequila, increases the concentration of furfural in the final product and reduces ethanol yield due to the caramelization of some of the fermentable agave sugars. This is why some factories with both cooking systems reserve the ovens for their better-quality products. Although it is easier to obtain well-cooked agave in an oven than in an autoclave, there is no difference in terms of flavor and fermentability between agave cooked in autoclaves or in ovens if both are correctly controlled.

Extraction of agave juice: milling

Milling has gone through three historical stages. In ancient days cooked agave was crushed with

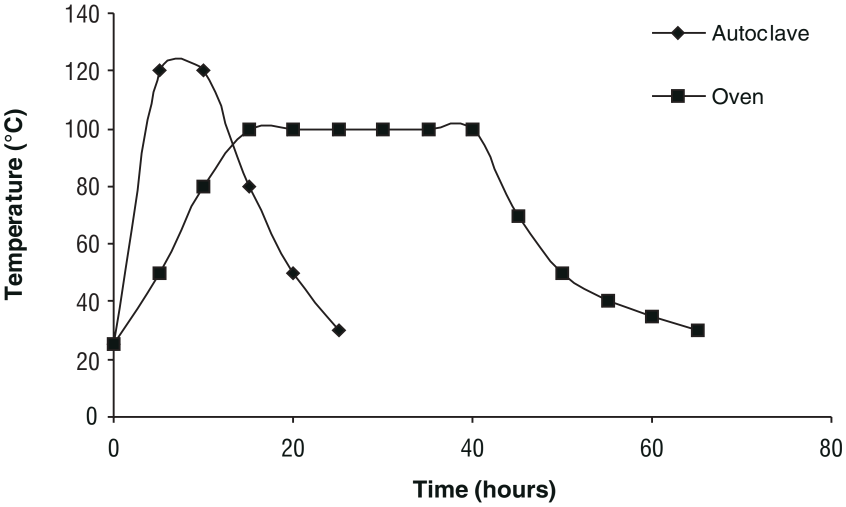


Figure 8. Temperature profile for agave cooking in oven and autoclave.

wood or steel mallets to extract the juice. Later, a rudimentary mill consisting of a large circular stone 1.3 m in diameter and 50 cm thick was used. Driven by animals, the stone turned in a circular pit containing cooked agave and extracted the juice. The resulting juice was collected by hand in wooden basins and carried to fermentation tanks. By the 1950s modern systems were implemented in which cooked agave was passed through a cutter to be shredded (except in factories that did this operation before cooking); and with a combination of milling and water extraction, sugars were extracted. The mills used for agave are similar to those used in the sugarcane industry but are smaller in size (normally 50 cm wide). This system is still employed in most distilleries.

In recent years the tequila industry has been using a technology to extract fructose in cooked agave or inulin in raw agave based on countercurrent extraction by means of a piece of equipment called a diffusion band. With the use of this technology, used for many years in the extraction of sugar and alcohol in grapes in Spain, the process has been improved and the bagasse at the end of the extraction has nearly no residual sugar.

Juice obtained in milling is mixed with the syrup obtained in the cooking step and with a solution of sugars, normally from sugarcane (if the tequila to be produced is not 100% agave), and finally pumped into a fermentor. Although the amount of sugar employed as an adjunct is regulated by law and must be less than 49% by weight at the beginning of the fermentation, each factory has its own formulation.

The milling step generates a by-product called bagasse, which represents about 40% of the total wet weight of the milled agave. Bagasse composition (dry weight basis) is 43% cellulose, 19% hemicellulose, 15% lignin, 3% total nitrogen, 1% pectin, 10% residual sugars and 9% other compounds. The bagasse, mixed with clay, is used to make bricks; but it is also the subject of research to find alternative uses. Examples are use of bagasse as an animal feed or as a substrate on which to grow edible fungi (Iñiguez *et al.,* 1990; 2001). Attempts are also underway to recover bagasse components (cellulose, hemicellulose and pectin) using high-efficiency thermochemical reactors (Alonso *et al.*, 1993), to obtain furfurals, make particle board, or enzymes (cellulase and pectinase). Many of these projects are at the laboratory stage, and there is not enough information to evaluate feasibility.

In milling, as in all steps of the tequila process, a sugar balance is computed to determine the yield. If the yield decreases, the extraction pressure in the mill and the water/agave ratio are increased to improve efficiency.

Fermentation

WORT FORMULATION

To produce 100% agave tequila, only agave may be used and the initial sugar concentration ranges from 4 to 10% w/v, depending on the amount of water added in milling. When other sugars are employed, they are previously dissolved and mixed with agave juice to obtain an initial sugar concentration of 8-16%, depending on sugar tolerance of the yeast strain. Wort formulation in most distilleries is based solely on previous experience. A few distilleries base wort formulation on composition of raw materials and nutritional requirements for yeast growth and fermentation. In these distilleries response surface methodology is the preferred method to optimize nutrient concentrations, using fermentation efficiencies and taste of the resulting tequila as responses (Montgomery, 1984). To correct nutritional deficiencies of agave juice and sugars employed in the growth and fermentation steps, urea, ammonium sulfate, ammonium phosphate or magnesium sulfate can be added. Because pH of the agave juice is around 4.5, there is no need for adjustment and the same wort composition is used for both inoculum growth and fermentation.

YEASTS

Some companies do not inoculate a specific strain of *S. cerevisiae* and instead allow natural fermentation to proceed. Others inoculate the wort with fresh packages of baker's yeast or a commercial dried yeast to obtain initial populations of 20-50 x 10^6 cells/ml. The dried yeasts were originally prepared for wine, beer, whisky or bread production; and sometimes the quality of the tequila obtained using these yeasts is not satisfactory, with large variations in flavor and aroma. To achieve high yields and maintain a constant quality in tequila, some companies use yeast strains isolated from a natural fermentation of cooked agave juice. Nutrients are added and special conditions such as a high sugar concentration or temperature are maintained. These isolated and selected yeast strains have been deposited in national microbial culture collections, the most important being the Biotechnology and Bioengineering Department Culture Collection of CINVESTAV-IPN, located In México City.

Toward the goal of improving fermentation productivity, one alternative is to use a strain of yeast capable of both efficiently converting wort sugars into alcohol and of producing the appropriate organoleptic compounds that impart a pleasant aroma to the final product. The difference in alcohol yield in tequila production using a selected commercial strain of *S. cerevisiae* compared with wild yeast is presented in Figure 9. The higher alcohol concentration at the end of the fermentation process clearly means better productivity and a lower production cost.

Fermentation temperature is important in tequila production since most of the distilleries are located in regions where ambient temperatures are normally warm, especially during the summer, and may be as high as 37ºC. Not all strains of *S. cerevisiae* are resistant to such high temperatures; and the selection of a commercial strain is very important as it can represent an advantage in process yield. Figure

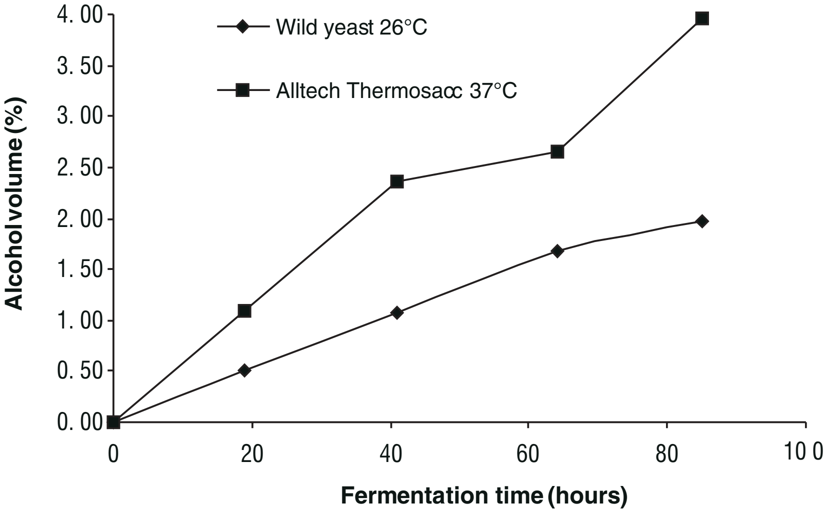


Figure 9. Alcohol concentration in a fermentation for tequila production using wild yeast compared with a commercial strain of yeast.

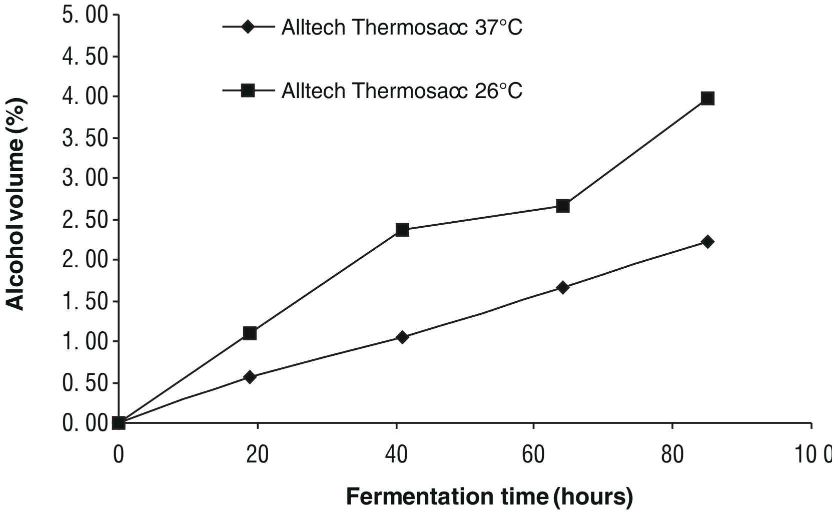


Figure 10. Alcohol concentration in tequila fermentation using a thermotolerant commercial strain of yeast at temperatures of 26 and 37ºC.

10 illustrates the fact that alcohol volume in the fermentation process for a thermotolerant strain is better at 37ºC than at 26ºC.

INOCULUM GROWTH

When an inoculum is used, it is grown in the laboratory from a pure culture of *S. cerevisiae* maintained on agar slants in lyophilized form or frozen in liquid nitrogen. All laboratory propagation is carried out under aseptic conditions using a culture medium with the same ingredients used in the normal process but enriched to promote cellular growth. The inoculum is scaled-up with continuous aeration to produce enough volume to inoculate fermentation tanks at 10% of the final volume. Populations of 200-300 x 10^6 cells/ml are normally achieved. Strict cleanliness is maintained in this step as bacterial contamination is highly undesirable. When contamination is detected, antibiotics or ammonium bifluoride are used as antimicrobial agents. Once an inoculum is grown, it is maintained by mixing 10% of the volume of an active culture with fresh agave juice and nutrients.

Although inoculation with commercial yeast greatly improves yield and turnover time, some companies prefer a more complex (in terms of the microbial diversity) fermentation. While yields might be lower and turnover time higher, the range of microorganisms produces more compounds contributing to a more highly flavored tequila. It is also important to recognize that a change in taste and flavor could negatively affect the market for a particular brand of tequila. For this reason it is very important that when a commercial strain of yeast is used in the fermentation process, the final aroma profile in tequila must be evaluated using a sensory evaluation panel together with gas chromatography analysis.

FERMENTATION OF AGAVE WORT

Once a wort is formulated with the required nutrients and the temperature is around 30°C, it may be inoculated with 5 to 10% (volume) of a previously grown *S. cerevisiae* culture with a population of 100-200 million cells/ml. Otherwise, microorganisms present in the wort carry out the fermentation. If an inoculum is not added, the fermentation could last as long as seven days. With an inoculum the fermentation time ranges from 20 hrs in the faster process to three days in the slower one.

Production of ethyl alcohol by yeast is associated with formation of many fermentation compounds that contribute to the final flavor of the tequila. These are organoleptic compounds or their precursors produced either in subsequent maturation of the wort before it goes to the distillation step, in the distillation process, or in the barrels if tequila is aged. The factors influencing formation of the organoleptic compounds in alcoholic beverages have been reviewed by many authors (Engan, 1981; Berry, 1984; MacDonald *et al.*, 1984; Ramsay and Berry, 1984(a); Geiger and Piendl, 1976). Experience in the tequila industry is that the amount of organoleptic compounds produced is lower in fast fermentations than in slow fermentations. As a consequence, the flavor and general quality of tequila obtained from worts fermented slowly is best. The rate of fermentation depends mainly on the yeast strain used, medium composition and operating conditions, as previously explained. The wort sugar content decreases from an initial value of 4-11% to 0.4% (w/v) reducing sugars if an efficient yeast strain is employed. Otherwise, the residual sugar content could be higher, increasing production costs.

Fermentation vessels vary considerably in volume, depending on the distillery. Capacity ranges from 12,000 liters for small tanks to 150,000 liters for the largest ones; and they are constructed of stainless steel in order to resist the acidity of the wort. Ethanol production can be detected almost from the onset; and a pH drop from 4.5 to 3.9 is characteristic of the fermentation. The alcohol content at the end of fermentation is between 4 and 9% v/v, depending on the initial sugar concentration. In order to increase the fermentation yield, in addition to selection of a good yeast strain, another option is the use of enzymes or enzyme complexes, to convert residual polymers from agave into fermentable sugars, which are converted mainly into alcohol improving the productivity of tequila production. Figure 11 shows the results of an experiment using

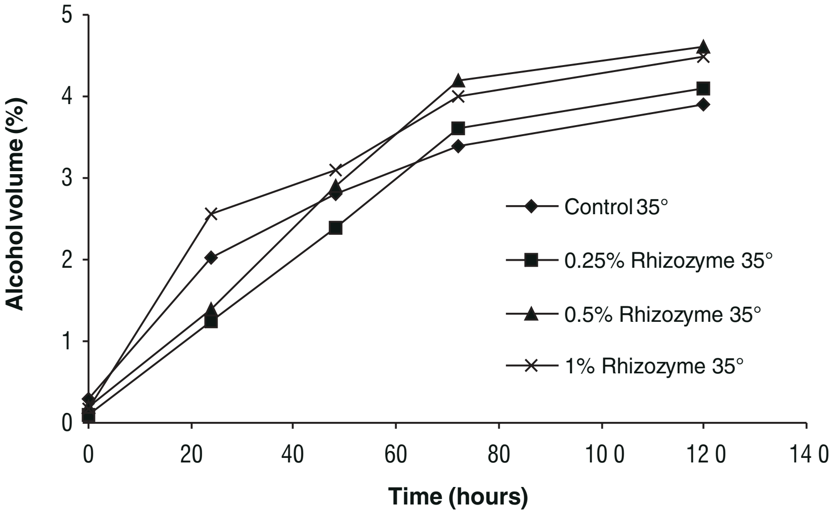


Figure 11. Alcohol volume in the wort at different concentrations of an enzyme complex (Rhizozyme™) for tequila production at 35ºC.

different doses of a commercial enzyme complex in the alcohol concentration of the wort.

Alcohol losses may be significant because many fermentation tanks are open, allowing evaporation of alcohol with carbon dioxide. Some of the largest distilleries have a cooling system that keeps fermentation temperature within a tolerable range for yeast, but small producers do not have these systems. The fermentation temperature can exceed 40°C, causing the fermentation to stop with an accompanying loss of ethanol and flavors that consequently decreases yields and affects the quality of the tequila. Fermentations carried out with pure agave juice tend to foam, sometimes requiring the addition of silica. In worts with added sugars, foaming is usually not a problem.

Non-aseptic conditions are employed in fermentation, and in consequence bacterial activity may increase. The size of the bacterial flora depends on a number of factors including the extent to which bacteria grow during yeast propagation (if used), the abundance of bacteria on the raw materials and hygiene standards in the distillery. There is no doubt that the activity of these bacteria contributes to the organoleptic characteristics of the final product. Occasionally, the size of the bacterial population in fermenting wort may become too large (>20 x 10^6 cells/ml), in which case the bacteria use the sugars, decreasing ethanol yields and sometimes excreting undesirable compounds. The same compounds used in the propagation step may be used here to decrease common bacterial contaminants found in tequila worts. *Lactobacillus, Streptococcus, Leuconostoc*, and *Pediococcus* are the most common contaminants, but *Acetobacter* may be found in fermented worts that are left inactive for a long time prior to distillation.

In contrast to other distilled beverages, the organoleptic characteristics of tequila come from the raw material (cooked agave) as well as from the fermentation process. In most of the processes used in the tequila industry, fermentation is spontaneous with the participation of microorganisms from the environment, mostly yeasts and a few bacteria. This peculiarity brings about a wide variety of compositions and organoleptic properties. However, the special characteristics of the wort make it a selective medium for the growth of certain yeasts such as *Saccharomyces* and to a lesser extent, acetic and lactic bacteria. In experiments isolating microbial flora from musts of various origins, different microorganisms were isolated. In most cases, this difference in flora is responsible for the wide variety of organoleptic characteristics of tequila brands (Pinal, 1999). Where a single purified strain is used, the final flavor and aroma are more neutral since the bouquet created by the contribution of several stains is richer than that obtained from only one type of yeast. Moreover, when yeast produced for bakery applications is used, the final product is also more neutral. It is also recognized that when non-100% agave tequila is made, a poorer bouquet is obtained because a more defined medium yields a more defined product.

ORGANOLEPTIC COMPOUNDS GENERATED DURING FERMENTATION

Fusel oil

As in many other alcoholic fermentation processes, higher alcohols are the most abundant compounds produced along with ethanol. We have found (in decreasing order of abundance) isoamyl alcohol, isobutanol, active isoamyl alcohol and phenylethanol. It has been established that production of isoamyl and isobutyl alcohols begins after the sugar level is lowered substantially and continues for several hours after the alcoholic fermentation ends. In contrast, ethanol production begins in the first hours of the fermentation and ends with logarithmic yeast growth (Pinal *et al.*, 1997).

The most important factor influencing the amount of isoamyl alcohol and isobutanol is the yeast strain. It was found that a native strain isolated from tequila must produces a higher amount of such compounds when compared with a strain usually employed in bakeries. These results agree with those reported for Scotch whisky (Ramsay and Berry, 1984(b)) and beer (García *et al.*, 1994).

The carbon:nitrogen ratio also has a significant influence on higher alcohol production. In tequila musts, which contain mainly fructose (≈95%) as a carbon source and an inorganic nitrogen source (ammonium sulfate), it was found for both native and bakery yeast strains that low carbon:nitrogen ratios result in low amounts of isoamyl alcohol: 19 mg/L in bakery strains and 30 mg/L for native strains *vs* 27 and 64 mg/L, respectively, for high carbon:nitrogen ratios. A similar relationship exists for isobutyl alcohol production (Figures 12 and 13).

Temperature is a third factor affecting isobutyl and isoamyl alcohol production with higher temperatures (e.g. 38 *vs* 32°C) yielding higher concentrations of those alcohols. On statistical analysis it was found that in addition to the direct effects of yeast strain, carbon:nitrogen ratio and temperature, the interaction of these three factors also had an impact on higher alcohol concentration in tequila (Pinal *et al.*, 1997). These results are consistent with the fact that with high carbon:nitrogen ratios there is a tendency to use amino acids as a nitrogen source, which implies the production of fusel oil as a by-product (by the Erlich pathway). On the other hand, variables such as the type of nitrogen source (urea or ammonium sulfate) or the amount of inoculum used for fermentation had

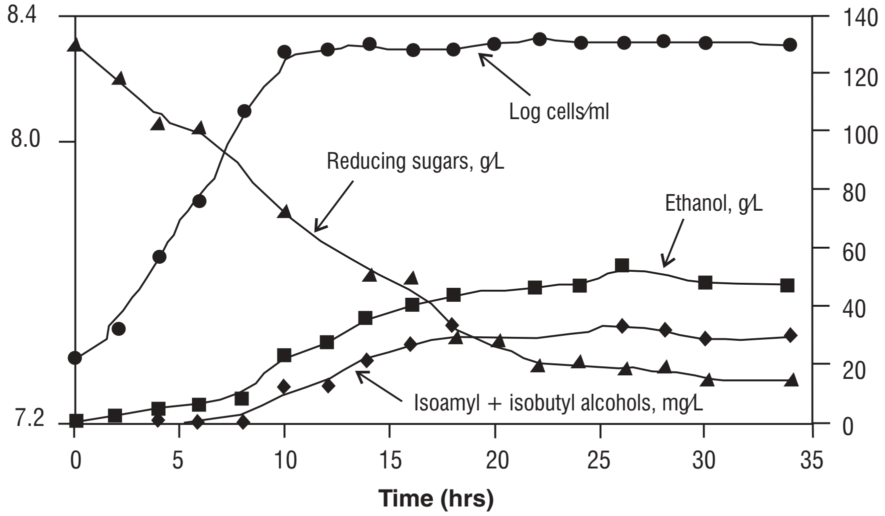


Figure 12. Higher alcohol production in tequila wort by a baker's yeast strain.

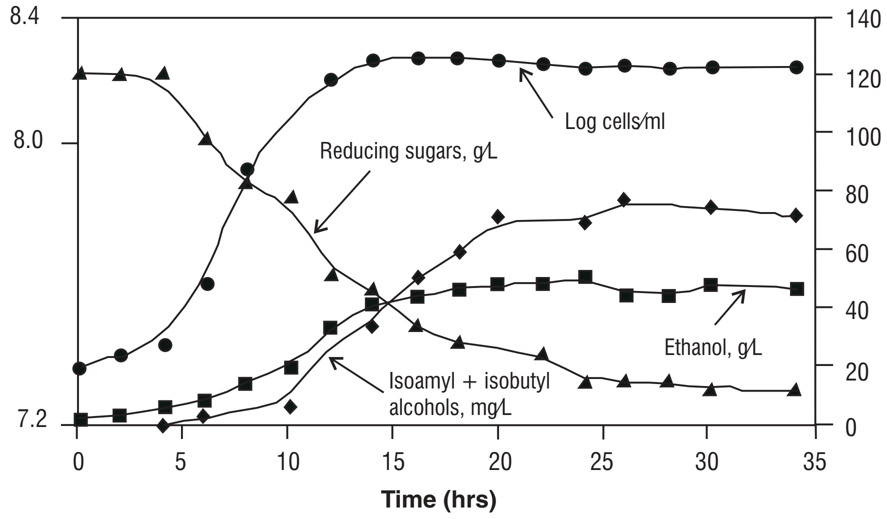


Figure 13. Higher alcohol production in tequila wort by a native yeast strain.

little or no effect on the production of higher alcohols. Pareto diagrams involving all the variables tested are depicted in Figures 14 and 15.

Methanol

Another characteristic compound present in tequila is methanol. It is generally thought that methanol is generated through hydrolysis of methylated pectins present in the agave plant. Nevertheless, it is also believed that some yeast strains, natural or inoculated, have pectin methyl esterases. Some authors describe various amounts of methanol in musts with the same composition but fermented with different strains (Téllez, 1999).

Aldehydes

Along with the production of ethyl acetate, the oxidation of ethanol also generates acetaldehyde, an intermediate in the production of acetic acid. It is well known in commercial practice that an oxidation process instead of fermentation begins after the sugar concentration declines, thus provoking the increase in acetaldehyde levels. However, there is no formal

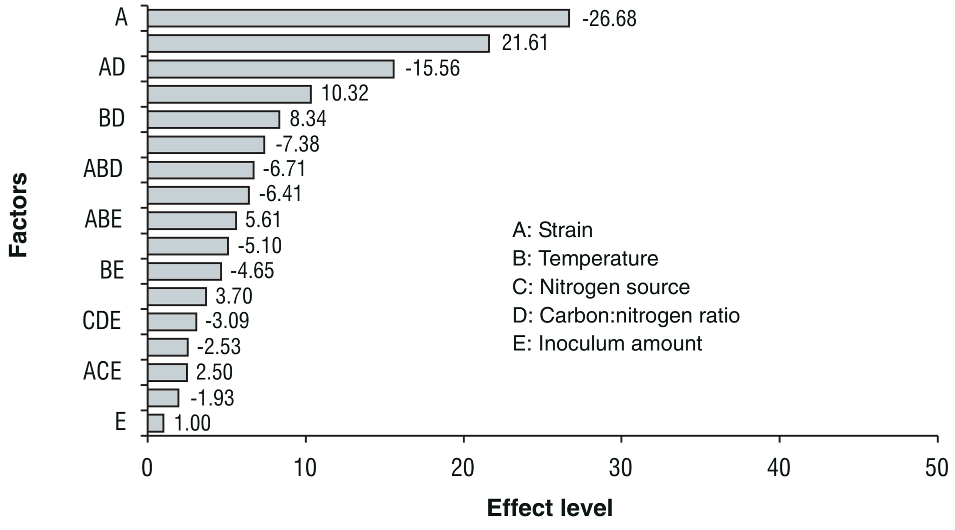


Figure 14. Pareto diagram for isoamyl alcohol production in tequila.

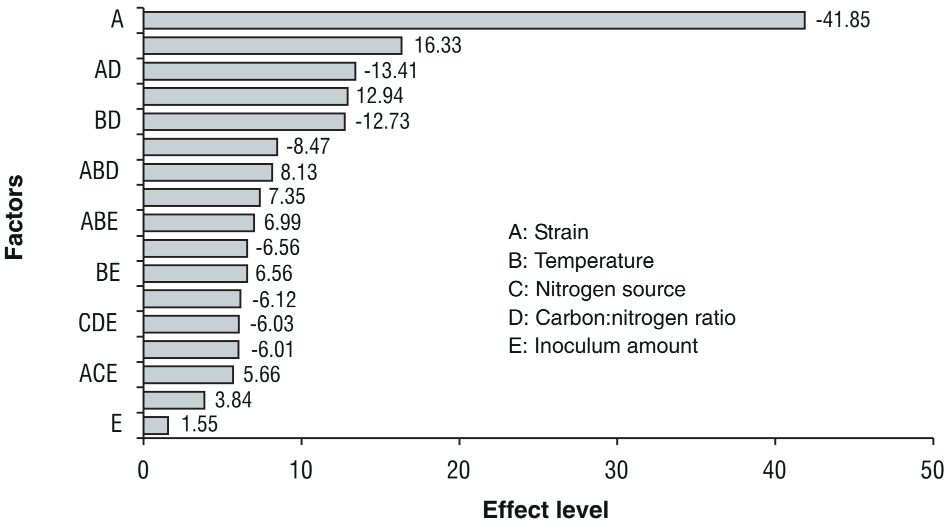


Figure 15. Pareto diagram for isobutyl alcohol production in tequila.

report of such phenomena in tequila, as is described in beer (Hammond, 1991).

Organic acids

Small organic acids (up to six carbons) and larger molecules (fatty acids) are produced during fermentation. The smaller molecules can be products of intermediate metabolism of the normal microbial flora; and their production depends on the presence of oxygen. The larger fatty acids are synthesized for membrane structures during cell growth and can also appear at the end of the fermentation when lysis takes place. The presence of octanoic and decanoic acids in the final product has been described particularly for tequila.

Esters

Esters are very important compounds in their particular contribution to flavor and aroma, since they have the lowest organoleptic threshold values (Ramsay and Berry, 1984a). In particular, ethyl acetate is the most abundant and important compound of this family. Ethyl acetate has been reported to be the second most abundant compound in tequila after isoamyl alcohol. The quantity of this compound present in the final product can vary widely, since it is synthesized from acetic acid (in the form of acetyl CoA) and ethanol. Acetic acid can also be produced by the oxidation of ethanol when the fermentation has ceased and an oxidative process starts on the surface of the fermentation tank by *Saccharomyces* and many other yeasts such as *Brettanomyces*. Therefore, long fermentation periods (a current practice in the tequila industry) yield high ethanol oxidation. In addition, in open fermentation tanks with worts at low pH containing alcohol, ethanol is also transformed to acetic acid (itself a precursor of ethyl acetate) by bacteria of the genera *Acetobacter*. Besides ethyl acetate, the presence of several other esters has been described including ethyl and isoamyl esters. Some of the most important esters found in silver tequila are listed in Table 2.

Table 2. Most abundant esters found in silver tequila.[1]

Ester	%
Ethyl acetate	17.77
Ethyl decanoate	2.78
Ethyl lactate	2.74
Ethyl octanoate	1.92
Ethyl dodecanoate	0.95
Ethyl butanoate	0.63
Isoamyl acetate	0.58
Ethyl propanoate	0.57
Ethyl hexanoate	0.48
Ethyl hexadecanoate	0.48

[1] Estarrón *et al.*, 1999.

Distilling

Distillation involves the separation and concentration of the alcohol from the fermented wort. In addition to ethanol and other desirable secondary products, wort contains solid agave particles consisting mainly of cellulose, pectin, and yeast cells in addition to proteins, mineral salts and some organic acids. Although many types and degrees of distillation are possible, the most common systems used in the tequila industry are pot stills and rectification columns. The pot still is considered the earliest form of distilling equipment. It is of the simplest design, consisting of a kettle to hold the fermented wort, a steam coil, and a condenser or a plate heat exchanger. Pot stills are often made of copper, which 'fixes', according to Thorne *et al.* (1971), malodorous volatile sulfur compounds produced during fermentation. Batch distillation using pot stills is carried out in two steps. First the fermented wort is distilled to increase the alcohol concentration to 20-30% by volume, separating out the first fraction called 'heads', and the last fraction, called 'tails'. Composition of these fractions varies depending on many factors including the yeast strain employed, wort nutrient composition, fermentation time and distillation technique; but in general, heads are rich in low boiling point compounds such as acetaldehyde, ethyl acetate, methanol, 1-propanol, 2-propanol, 1-butanol, and 2-methyl propanol, which give a very pleasant flavor and taste to tequila. Heads are normally mixed with the wort being distilled. The tails contain high boiling point components such as isoamyl alcohol, amyl alcohol, 2-furaldehyde, acetic acid and ethylactate, giving a strong taste and flavor to the tequila; and when the concentration is above 0.5 mg/ml, the final product becomes unpleasant. This fraction is not used.

In the second step, the liquid obtained from the first stage is re-distilled in a similar pot still in order to obtain a final product that is 110° proof if it is sold in bulk (reducing transport costs) or 80° proof if it is to be bottled. Some companies obtain high proof tequila and dilute it with demineralized water or water purified by reverse osmosis.

In continuous distillation systems, the fermented wort enters the feed plate of the column and flows downward, crossing a series of trays. Steam is injected from the bottom in a coil and strips the wort of its volatile components. Vapors condense higher in the column, depending on component volatility, allowing liquids to be drawn off or recycled at the various plates as appropriate. Sometimes tequila obtained in this way is mixed with tequila from pot stills to balance the amount of organoleptic compounds because in general, tequila obtained through continuous columns has less aroma and taste than tequila obtained from pot stills.

The presence of methanol in tequila is still a subject of discussion because whether methanol is produced only by a chemical reaction or in combination with a microbial hydrolysis has not been satisfactorily demonstrated. The chemical reaction is demethylation of agave pectin by the high pH during cooking and the first distillation steps. The microbial reaction could be the hydrolysis of agave pectin by the enzyme pectin methyl esterase produced by some microorganisms during the fermentation step, but this has not yet been demonstrated. Preliminary results favor the first theory, but research is still needed on this matter because it is important to maintain the methanol concentration within the limits established by the official standard, which is 300 mg per 100 ml of anhydrous ethyl alcohol. Table 3 shows the specifications for tequila according to the standard of quality from the Mexican Official Norm (Secofi, 1994).

Effluent disposal

The discharge from pot stills or distillation columns is known as stillage, slops or vinasse; and in a typical tequila distillery 7 to 10 liters of effluent are produced per liter of tequila at 110º proof. Tequila stillage has a biological oxygen demand (BOD) of 25 to 60 g/L. In addition to the dissolved salts (mainly potassium, calcium, and sulfate ions) and the low pH (<3.9) of the stillage, there are significant disposal or treatment problems. A general solution to the disposal problem does not exist because every factory has its own production process and is located either in a city or near agave fields. As a result of the difficulties of treating vinasses and due to

Table 3. Tequila specifications according to NOM.

	Tequila blanco (White tequila)		*Tequila joven u oro (Gold tequila)*		*Tequila reposado (Aged)*		*Tequila añejo (Extra-aged)*	
	min	*max*	*min*	*max*	*min*	*max*	*min*	*max*
Alcohol, % bv at 20ºC	38.0	55.0	38.0	55.0	38.0	55.0	38.0	55.0
Dry extract, g/L	0	0.20	0	5.0	0	5.0	0	5.0
	Values expressed as mg/100 ml pure ethanol							
Higher alcohols (as amyl alcohol[1])	20	400	20	400	20	400	20	400
Methanol[2]	30	300	30	300	30	300	30	300
Aldehydes	0	40	0	40	0	40	0	40
Esters	2	270	2	350	2	360	2	360
Furfural[3]	0	1	0	1	0	1	0	1

[1] Using a Gas Chromatograph the value could be up to 500 mg/100 ml.

[2] Minimum could be less than this value if the distillery could demonstrate the use of a technology to reduce methanol concentration.

[3] Using chemical analysis the maximum value could be up to 4 mg/100 ml.

their high concentrations of dissolved matter, a host of utilization schemes have been proposed. Some of the methods indicated below are under investigation and others are in use.

Recycling reduces the volume of waste to be treated. Stillage can be recirculated, mixing 5 to 10% of the total volume of the waste obtained with clean water to substitute for the dilution water used to prepare the initial wort. This can be carried out for a number of cycles, usually no more than five, because the concentration of dissolved salts increases and could affect the fermentation process. Also, great care must be taken with the final taste and flavor of the tequila because some components present in stillage could affect the organoleptic characteristics of the final product. Currently, only one tequila company uses this system.

Direct land application as irrigation water and fertilizer in agave fields is under careful evaluation to determine the optimum loading rates and the effects on the agave over the long time needed to reach maturity. Evaporation or combustion of stillage could provide fertilizer or potash, but the high cost of such a process is a serious limitation (Sheenan and Greenfield, 1980). The production of biomass and biochemicals including fodder yeast is a possibility (Iñiguez *et al.*, 1996), but the remaining liquor still has a high BOD (Quinn and Marchant, 1980). Stillage may be used as a food supplement for cattle, but it has an undesirable laxative effect on animals. Biological, aerobic, or anaerobic treatment offers a real means of disposal, but the cost is likely to be as high as the fermentation costs themselves (Speece, 1983; Maiorella, 1983). Ultimately, tequila vinasses should be viewed as a raw material rather than a waste, and a strategy should be devised that maximizes economic and social benefits and reduces recovery costs.

Maturation

Distillation is the final stage of tequila production if silver or white tequila is the desired product. For rested (reposado) or aged (añejo) tequila, maturation is carried out in 200 liter white oak casks or in larger wood tanks. The time legally required is two months for rested tequila and 12 months for aged tequila. Tequila is generally matured for longer periods, depending on the characteristics each company desires for its particular brand.

As tequila ages in barrels, it is subject to changes that will determine its final quality. Thickness and quality of the stave, depth of the char, temperature and humidity in the barrelling area, entry proof (40-110° proof), length of storage and number of cycles for the barrel (in Mexico barrels may be re-used several times) all affect the final taste and aroma of the tequila. Fusel oil content decreases during maturation owing to the adsorbant nature of the char, smoothing the final product. Complex wood constituents are extracted by the tequila, providing color and the particular taste. Reactions among certain tequila compounds yield new components; and oxidation reactions change some of the original components in tequila and those extracted from the wood. As a result of all of these changes, the concentration of acids, esters and aldehydes is increased, while the concentration of fusel oils decreases as tequila reposes in barrels.

After aging and dilution with demineralized water (if necessary) the color of the tequila may be adjusted to the desired value by the addition of caramel. Alternatively, some companies blend different batches of tequila to obtain a standardized final product.

Government inspectors supervise the entire aging process. Prior to bottling, tequila is filtered through cellulose filter pads or polypropylene cartridges. Sometimes afterwards a pretreatment with charcoal is used to eliminate turbidity.

Production statistics

Tequila production has grown since 1995 at an average rate of more than 10% annually and reached its maximum production of 190.6 million liters of tequila (at 40% alcohol by volume) in 1999 (Figure 16). After that, a shortage of agave occurred due to a combination of environmental and market factors. A bacterial infection (*Erwinia carotovora)* and a fungus (*Fusarium oxysporum*), affected agave harvests, while the growing number of distilleries, development of many new brands in the market, and an increase in tequila consumption in the domestic and export market affected demand. The drop in production began in 2000 with a reduction to 181.6 million liters and in 2002 declined to 141 million liters.

Production of 100% agave tequila had the most pronounced decrease due to the agave shortage, because 6.0 kg of agave are required to produce 1 liter of tequila (at 55% abv) whereas other tequilas require only 3.0 kg of agave per liter. Most tequila producers moved into production of other tequila types and reduced amounts of 100% agave tequila.

The outlook for the future appears promising for tequila production, and a return to previous industry growth rates is expected by the end of 2003.

In the export market the scenario was very similar but the effect of the agave shortage lasted through 2002 since the main export companies sell tequila in bulk (Figure 17). Total export volume was at its lowest in 2001 (75.6 million liters of tequila at 40% abv) with the beginning of recovery noted in 2002. Exports of 100% agave tequila became almost stable at a volume

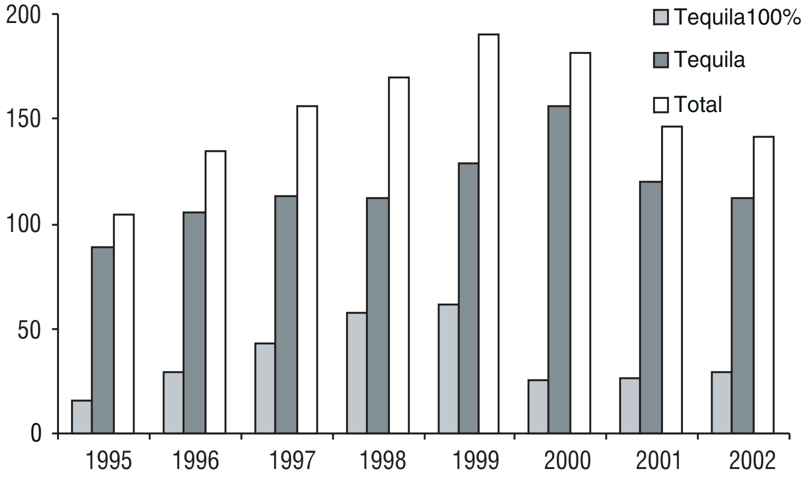


Figure 16. Tequila production in Mexico in millions of liters at 40% abv (CRT, 2002).

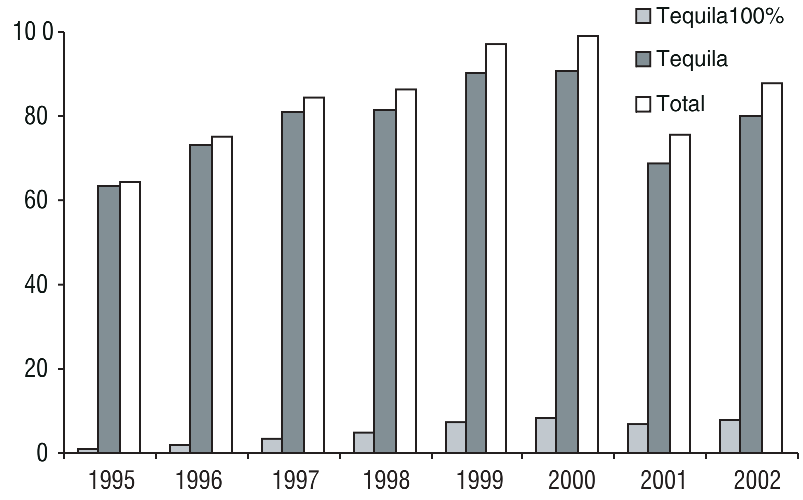


Figure 17. Exports of tequila by type in million liters at 40% abv (CRT, 2002).

near 8 million liters. Again, it should be noted that this type of tequila can only be exported bottled and not in bulk.

Although the European and other markets are growing, nearly 80% of all tequila exported continues to go to the US market with volumes that in 2002 represented over 69 million liters of tequila at 40% abv, which is more than the 53 million liters consumed in the domestic market (Figure 18). Finally, even though only 25% of the tequila is exported bottled during 2002 (22 million liters of tequila at 40% abv), this has been growing since 1995 when 6.5 million liters were state-bottled (Figure 19).

Future developments

Research and future developments in the production of tequila and agave cultivation can take many directions, but the implementation of any change must allow quality of the final

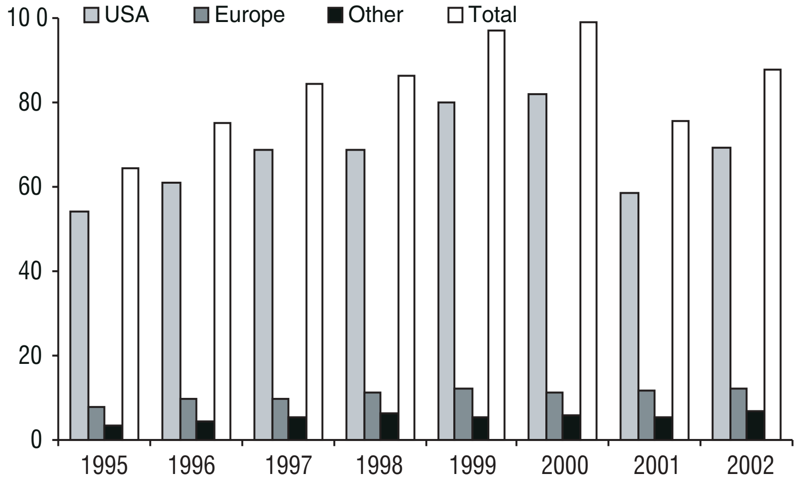


Figure 18. Exports of tequila by destination in million of liters at 40% alcohol in volume (CRT 2002).

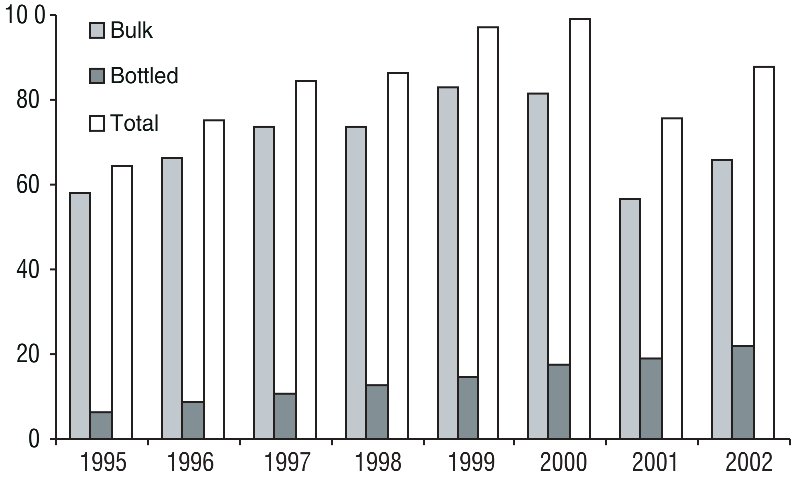


Figure 19. Exports of tequila in millions of liters at 40% abv (CRT, 2002).

product to be maintained and provide substantial improvement in the process. There are several key areas where important developments could take place. Development of new varieties of agave endowed with resistance to pests or extremely dry environments, use of micropropagation techniques, satellite remote inspection to evaluate health status of agave plantations, higher inulin content, low wax content in the leaf cuticle and superior growth rates would all be of benefit. Mechanization would improve aspects of cultivation and harvest of agave. In addition, optimization of the cooking and fermentation steps would improve yields and reduce the amount of waste, while yeast strain selection could improve ability to ferment musts with high concentrations of sugars. Another area of opportunity is the use of isotope (H^2 and O^{18}) measurement in tequila to authenticate the beverage. Finally, low cost alternatives for waste (bagasse and stillage) treatment are needed.

Summary

Tequila is a beverage obtained from the distillation of fermented juice from the agave plant *(Agave tequilana.* Weber var. azul). The use of agave to produce drinks in Mexico dates from long ago when certain tribes used them for religious ceremonies. It was not until the arrival of the Spaniards, who brought knowledge of distillation techniques, that tequila acquired its present form. The agave plant, which is often confused with cacti, is propagated through traditional methods or by means of plant cell culture. It is grown for 7 to 8 years before it can be harvested and sent to the distillery.

The tequila production process comprises five stages. In the first step, agave is cooked to hydrolyze the polymers present in the plant, mainly inulin, into fermentable sugars. In some factories this step is accomplished using stone ovens and in others it is carried out in autoclaves. The second stage is sugar extraction from cooked agave through milling; and the agave juice obtained in this step can be mixed with sugars from other sources, normally sugar cane, if 100% agave tequila is not desired. The third and most important stage is fermentation in which sugars are transformed into ethanol and other compounds such as esters and organic acids. These, along with other substances derived from the cooked agave, give the characteristic flavor and taste to tequila. It is of great importance to have a good yeast strain and nutritionally balanced wort for tequila production, as losses can be as high as 35% of the total production if inefficient yeast is used or nutrients are not present in the right proportions. In the fourth stage, fermented wort is distilled, normally using pot stills or in some cases rectification columns, to obtain the final product. At the end of the distillation process white tequila is obtained. Maturation, the last stage, in white oak barrels is required for rested or aged tequila. The minimum maturation times are 2 and 12 months, respectively, for rested and aged tequila as required by government regulations. At every step of the production process, most companies employ several quality control analyses in order to ensure the quality of the product and the efficiency of the process. Some major producers of tequila are certified through an ISO-9000 standard. Tequila production is governed by the official norm NOM-006-SCFI-1994, which must be followed by all tequila producers to guarantee a good quality final product.

Acknowledgement

The author would like to express his gratitude to Tequila Herradura, S.A. de C.V., for its support in writing this chapter.

References

Agricultural Research Service. 1972. The Agave family in Sonora. *Agriculture Handbook No.399,* US Department of Agriculture, p. 195.

Alonso, G.S., L. Rigal and A. Gaset. 1993. Valoración química de bagazo de agave de la industria tequilera. Rev. Soc. Quim. Mex. 3(6):19.

AOAC 1990. Distilled liquors. Official Methods of Analysis of the Association of Official Analytical Chemists (H. Kenneth, ed) Association of Official Analytical Chemists, Arlington, Virginia, p. 690.

Backman, G.E. 1944. A karyosystematic study of the genus Agave. Am. J. Bot. 35:283.

Berry, D.R. 1984. Physiology and microbiology of the malt whisky fermentation. In: *Progress in Industrial Microbiology*. (M.E. Bushell, ed.) Elsevier, Amsterdam, p. 189.

Bottorff de B., V. 1971. A guide to tequila, mezcal and pulque. Minutae Mexicana, Mexico, D.F.

Casado, C.G. and A. Heredia. 2001. Self-association of plant wax components: a thermodynamic analysis. Biomacromolecules 2:407-409.

CRT. 2002. Consejo Regulador del Tequila. Informe de Actividades.

Diguet. L. 1902. Estudio sobre el maguey de tequila. Generalidades e historia. El Prog. Mex. 9:424.

Ehrler, W.H. 1967. Agave plant, efficient water user. USDA Agr. Res. 16(4):11.

Engan, S. 1981. Beer composition: volatile substances. In: *Brewing Sciences*, Vol. 2 (J.R.A. Pollock, ed.) Academic Press, London, p. 98.

Estarrón, M., T. Martín del Campo and R. Cosío. 1999. Identificación de los componentes volátiles que caracterizan la huella cromatográfica distintiva de tequilas. Technical Report for Tequila Herradura S.A.

García, A.I., L.A. García and M. Díaz. 1994. Fusel alcohol production in beer fermentation processes. Process Biochem. 29:303-309.

GEA. 1992. La fertilización, La Jima. *Boletin Informativo,* Gerencia de extensión agrícola. Tequila Sauza, 7(50).

Geiger, E. and A. Piendl. 1976. Technological factors in the formation of acetaldehyde during fermentation, MBAA Tech. Q. 13:51.

Goncalves de L. O. 1956. El Maguey y el Pulque, Fondo dc Cultura Económica, México.

Granados, S.D. 1985. Etnobotanica de los agaves dc las zonas áridas y semiáridas. In: *Biología y Aprovechamiento Integral del Henequén y Otros Agaves*, CICY, A.C., México, p. 127.

Halffter, G. 1975. Plagas que afectan a las distintas especies de agave cultivado en México. Secretaría de Agricultura y Ganadería, México, D.F.

Hammond, J.R.M. 1991. Brewer's Yeast. In: *The Yeasts*, Vol. 5. Yeast Technology (A.H. Rose and J.S. Harrison, eds.) 2nd. ed. Academic Press, Redding, U.K.

Iñiguez, C.G., I.J.A. Cuaron, G.P. Perez, M.M. De la Torre and P.I. Magaña. 1990. Fermentation characteristics, digestibility and performance of ensiled swine waste, wheat straw and cane molasses fed to sheep. Biol. Wastes 34(4):281-299.

Iñiguez, C.G., G.Ma. de J. Franco and O.G. Lopez. 1996. Utilization of recovered solids from tequila industry vinasse as fodder feed. Bioresource Technology 55(2):151-155.

Iñiguez, C.G., S.E. Lange and R.M. Rowell. 2001. Utilization of byproducts from the tequila industry: Part 1: Agave bagasse as a raw material for animal feeding and fiberboard production. Bioresource Technology 77:25-32.

Luna, Z.R. 1991. La historia del tequila, de sus regiones y de sus hombres. Consejo Nacional para la Cultura y las Artes, México, D.F.

MacDonald, J., P.T.V. Reeve, J.D. Ruddlesden and F.H. White. 1984. Current approaches to brewery fermentations. In: *Progress in Industrial Microbiology*. Vol. 19, Modern Applications of Traditional Biotechnologies, (M.E. Bushell, ed.) Elsevier, Amsterdam.

Maiorella. B.L., H.W. Blanch and C.R. Wilke. 1983. Distillery effluent treatment and by-product recovery. Proc. Biochem. 8(4):5.

Mendez, R.M.D. 1999. Acumulación de fructanas en *Agave tequilaza* Weber var. azul cultivado en campo. MS Food Science Tesis. ENCB-IPN pp. 80-87.

Montgomery, D.C. 1984. *Design and Analysis of Experiments*, 2nd Ed., John Wiley & Sons, New York. p. 445.

Muria, J.M. 1990. El tequila. Boceto histórico de una industria. Cuad. Difus. Cient. 18:13.

Pérez, L. 1990. Estudio sobre el maguey llamado Mezcal en el estado de Jalisco. Programa de Estudios Jalisciences, Instituto del Tequila, A.C., México.

Pinal, Z.L.M. 1999. Tesis de Maestría en Procesos Biotecnológicos. Universidad de Guadalajara, Guadalajara, México.

Pinal, Z.L.M. 2001. Influencia del tiempo de cocimiento sobre la generacion de compuestos organolepticos en las etapas de cocimiento y fermentación en la elaboración de tequila. MS Biotechnology Tesis. U. de G. Cucei pp. 85-91.

Pinal, L., M. Cedeño, H. Gutiérrez and J. Alvarez-Jacobs. 1997. Fermentation parameters influencing higher alcohol production in the tequila Process. Biotechnol. Lett. 19(1):45-47.

Pla, R. and J. Tapia. 1990. El agave azul, de las mieles al tequila. In: *Centro de Estudios Mexicanos y Centroamericanos, Instituto Francés de America Latina y el Institut Francais dc Recherche Scientifique pour le developement en Cooperation*, *México*, p. 61.

Quinn, J.P. and R. Marchant. 1980. The treatment of malt whisky distillery waste using the fungus *Geotrichum candidum*. Water Res. 14:545.

Ramsay, C.M. and D.R. Berry. 1984a. The effect of temperature and pH on the formation of higher alcohols, fatty acids and esters in malt whisky fermentation. Food Microbiol. 1:117.

Ramsay, C.M. and D.R. Berry. 1984b. Physiological control of higher alcohol formation in Scotch whisky fermentation. In: *Current Developments in Yeast Research* (G.G. Stewart and I. Russell, eds) Pergamon Press, London, Canada.

Rzedowski, J. 1978. *Vegetación de México,* Ed. Limusa. México.

Sánchez, A.F. 1991. Comparación de metodologías de micropropagación de *Agave tequilana* Weber. Tesis de Ing. Agrónomo, Facultad de Agronomía, Universidad de Guadalajara, Guadalajara, México.

Sánchez-Marroquín, A. and P.H. Hope. 1953. Fermentation and chemical composition studies of some species. Agric. Food Chem. 246:1.

Secofi. 1994. Norma Oficial Mexicana. NOM-070-SCFI-1994. Bebidas alcoholicas-Mezcal-Especificaciones. Diario oficial 4 de Julio.

Sheenan, G.J. and P.F. Greenfield. 1980. Utilization, treatment and disposal of distillery wastewater. Water Res. 14:257.

Speece, R.E. 1983. Anaerobic biotechnology for industrial wastewater treatment. Environ. Sci. Technol. 17(9):416A.

Téllez, P. 1999. Tesis de Maestría en Procesos Biotecnológicos. Universidad de Guadalajara, Guadalajara, México.

Thorne, R.S.W., E. Helm and K. Svendsen. 1971. Control of sulfur impurities in beer aroma, J. Inst. Brew. 77(2):148.

Valenzuela, Z.A. 1992. Floración y madurez del agave, La Jima. Boletin Informativo, Gerencia de extensión agrícola. Tequila Sauza 5:33.

Chapter 16

Production of heavy and light rums: fermentation and maturation

ROBERT PIGGOT

Alltech Inc., Nicholasville, Kentucky, USA

Rum defined

Rum differs from Scotch, bourbon or cognac in that it is produced in many countries. In fact many countries import bulk rum, mature and blend it then market it either locally or internationally. The country where it is sold sets the definition of rum, which means that it is not the producing country that defines the ingredients and processes involved in making rum.

In the US the BATF definition of rum is*:*

> *"An alcoholic distillate from the fermented juice of sugar cane, sugar cane syrup, sugar cane molasses, or other sugar cane by-products, produced at less than 190° proof in such manner that the distillate possesses the taste, aroma and characteristics generally attributed to rum, and bottled at not less than 80° proof; and also includes mixtures solely of such distillates."*

The EU definition is essentially the same:

> *"A spirit drink produced exclusively by alcoholic fermentation and distillation, either from molasses or syrup produced in the manufacture of cane sugar or from sugar cane juice itself, and distilled at less than 96% vol, so that the distillate has the discernible specific organoleptic characteristics of rum."*

The two key points in both these definitions is that rum must be made with products derived from sugar cane (beet molasses products cannot be called rum and have a very different flavor profile) and it must have taste and aroma associated with rum. This part of the definition has been stretched in recent years with the very light white rums. In some blended drinks the spirit is referred to as 'molasses spirit' instead of rum for this reason. These definitions may appear vague, which is due to the wide range of styles and production methods that go into producing quality rum. For this reason this chapter is intended to give an overall guide to rum production and should be read in conjunction with the chapters on molasses as a feedstock and beverage alcohol distillation also in this volume.

Types of rum

Rum, along with its sister spirits such as cachaça (ka-shah'-sa) from Brazil (1.3 billion litres produced per annum according to Brazilian government figures), cane spirit from South Africa as well as much of the world's vodka spirit are all made from sugar cane. This means that a large percentage of the world's beverage alcohol is made from the cane grass. Rum can be loosely divided into three groups: dark, amber and white. *White rum* is produced by continuous distillation. It is not aged, except where required by law. When aged, it is aged in old well-used barrels and charcoal treated before bottling. It has a light and delicate nose and flavor. *Amber rum* is aged in wood for a short period, from six months to two years. Amber rum may contain some pot still rum for flavor. *Dark rum* is more fully bodied. It is aged in wood for longer periods, up to 12 years.

History

The beginnings of rum go back to the mid 1600s, possibly originating in Barbados, where it was known as a 'hot and hellish' drink. It carried nicknames such as 'kill devil' and 'rumbullion', the latter meaning clamor or noise. Today rum is associated with warm tropical islands, images of swashbuckling pirates and the British Navy. The link between rum and the British Navy runs deep:

- Vice Admiral William Penn first gave it in 1655 after the capture of Jamaica, which did not have brewing facilities. It gradually replaced beer as it could be stored for longer periods.
- It became the official drink of the BritishNavy in 1731; and each sailor received daily a half pint (tot) of undiluted rum. The previous ration was one gallon of beer or wine a day. In their days, Hawkins and Frobisher said they could cruise as long as the beer lasted.
- In order to reduce drunkenness among the crew, on August 21, 1740 Vice Admiral Vernon ordered the daily rum ration diluted with water. The admiral's nickname, Grog, was given to the diluted mixture.
- After the battle of Trafalgar in 1805 the body of Lord Nelson was preserved in rum, giving it the nickname 'Nelson's Blood'.
- The tot was reduced to 1/8 pint on January 1, 1851.
- July 30, 1970 **'Black Tot Day'** was the last day the traditional tot was given in the British Navy.

Dark rum was the only type of rum available for many years. It was generally built on the image of British Navy Rum. As tastes changed it declined in popularity. The Bacardi revolution of the 1960s, with its amber and light rums, quality control and marketing changed the image of rum and helped make it the popular beverage it is today. Even quality dark rum, which has all the nuances of a fine cognac, is making a strong comeback. Today the rum industry employs 50,000 people in the Caribbean basin and generates $260,000,000 in export earnings.

Equipment and feedstocks

EQUIPMENT

Rum is made with a wide variety of equipment, both old and modern. Until recently, an original wood-sided Coffey still was still in use in Guyana. The art of fine rum making is not in the equipment; it is in understanding and operating the equipment to produce the desired characteristics.

SUBSTRATES

Molasses

The use of molasses as a feedstock for alcohol production has been covered in an earlier chapter in this volume; therefore only a few comments are necessary with specific reference to rum.

The source of the molasses can have a strong influence on the aromatic quality of rum. This has been demonstrated in trials on making rum from beet molasses which showed that it was not possible to obtain the same characteristic aromas as obtained from cane molasses (Arroyo, 1948). Arroyo (1941, 1942) reported that fresh blackstrap molasses with low viscosity, high total sugars, nitrogen, phosphorus and a low ash and gum content was preferable in the production of rums with desirable odors and tastes. Good molasses for rum production should be low in ash and sulfur and high in fermentable sugars and free amino nitrogen.

Cane juice

Most rum is produced from blackstrap molasses, although cane juice is used in the French Caribbean islands. This spirit, known as 'agricultural rum' has a very high congener value (over 2,250 ppm) (EU regulations). Using cane juice for rum production has both advantages and disadvantages. The main advantage is that no additional processing is required. The cane is simply crushed and the juice fed directly into the fermentors. Another advantage is that cane juice does not have a high content of dissolved salts and therefore does not cause as much scaling and blocking of distillation columns as may occur using molasses.

One of the main disadvantages of using cane juice is that it normally contains only about 12-16% w/w sugar, so that the alcohol content of the fermented material is limited to about 6-8% v/v as compared to 10% v/v obtainable from molasses. Another significant disadvantage is that cane juice cannot be stored in bulk. Cane juice normally becomes heavily infected with bacteria and yeast in the crushing process. This contamination may significantly reduce alcohol yields and may cause it to start to ferment spontaneously and very rapidly. This means that the distillery must be located in close proximity to the cane mill. It also means that the cane juice is only available during the cane-crushing season, which generally does not exceed six months per year. Cane juice may also have cost disadvantages in that it is a primary, or at least an intermediate product in the production of sugar. Sugar production is usually heavily subsidized and so it is to the producer's benefit to produce as much as possible. In contrast, molasses is generally a by-product of sugar production, considered to be of lower value. Cane juice can be blended with molasses to improve the FAN content and reduce the amount of ash.

Fermentation and yeast

HEAVY RUM

Flavors present in the final product are produced during fermentation. Distillation refines the flavor, but does not create the base components. In order to get the desired flavor profile many companies use their own yeast culture, which they grow up from a slant. This yeast must be grown under sanitary conditions with a small quantity of sterile air and yeast food to establish healthy yeast growth. The solution is usually a molasses solution of about 16°Brix. The volume of the final propagator should be about 10% of the fermentor volume and have a cell count of at least 200 million cells/ml (Murtagh, 1999). In practice the yeast count in the propagation phase is often lower than this. It is important that the yeast in the propagator is still in the exponential growth phase when added to the fermentor. If kept too long, the yeast will enter the stationary phase and bacteria present in the fermentor will flourish on the sugar present. Some rum producers rely on the fermentation to start spontaneously from naturally occurring flora. This procedure results in a relatively inefficient fermentation with low yields and varying quality. The high level of bacterial contamination in these fermentations results in many desirable congeners, especially acids and esters. Jamaica in particular is renowned for its production of high ester rums. Most of these are used as blending stock to add flavor to dark and amber rum.

Arroyo (1945) has shown that the best rum yield and aroma were achieved with a yeast:bacteria ratio of 5:1. In order to create a non-yeast congener profile in a more controlled manner, a pure culture of bacteria such as *Clostridium saccharobutyricum* (its presence has been shown to accelerate fermentation (Arroyo, 1945)) may be added after 6-12 hrs of the yeast fermentation. Usually the bacterial inoculum amounts to about 2% of the fermentor capacity; and the pH of the fermenting mash is adjusted upwards to about pH 5.5 before addition to give more suitable conditions for the bacterial propagation. The bacteria produce a mixture of acids, predominantly butyric, together with others such as acetic, propionic, and caproic acids. These acids in turn react with the alcohol to produce desirable esters (Murtagh, 1999).

FERMENTATION FOR LIGHT RUMS

In light rum production, the emphasis is generally on maintaining clean, rapid fermentations to minimize development of undesirable congeners and to maximize fermentation efficiency. Many distilleries use commercial distillers yeast. Good yeast for the production of light rum should be tolerant of high temperatures (most rum is made in the tropics and the fast fermentations make cooling especially difficult), fast (to use the sugars before bacteria metabolize them thereby reducing yield), tolerant of high osmotic pressure and resistant to acid stress. The yeast selected should also provide a light congener profile. Unlike whole grain fermentations, it is possible to recycle the yeast from molasses fermentations either by flocculation or centrifugation of the dead wash. Traditionally recycled yeast has been 'acid washed', i.e., reduced to a pH of about 2.3 using phosphoric acid and added to the next fermentor; or it may be treated with antimicrobials, or with about 15 ppm Cl from chlorine dioxide at a pH of about 3.5 (Murtagh, 1999). The objective of yeast recycling is to take advantage of sugar that would otherwise be needed for yeast growth and to ensure a very high cell count in the fermentor inoculum. Recycling of yeast is generally not recommended. The yeast is in the stationary phase, and therefore 30 times the amount of yeast must be added in order to get the same rate of alcohol production as fresh budding yeast. Also it has been shown that bacteria can become resistant to acid washing, so bacteria are also getting recycled. It is recommended that when recycled yeast is used it is dumped and renewed on a regular basis.

Distillation

The method of distillation used has a considerable effect on the nature of the rum product. Heavy rums are usually produced by batch distillation or in a 2 column continuous still, while light rums are normally produced by continuous distillation with a hydrofining column.

POT DISTILLATION

There are a wide variety of pot stills in use in the rum industry, which vary from the straight pot still to the pot still with a distillation head as described in the chapter on beverage alcohol distillation. One type of pot still common in the Caribbean rum industry is the double retort (Figure 1). This still can be operated either slowly with very low steam input or quickly with a high steam input. The still is made of copper and should be inspected on a regular basis as the copper takes part in the chemical reactions during the distillation process. Wash at about 8% abv is pumped into the pot. The steam vaporizes this mixture; and the vapor is approximately 50% abv. Some of this condenses in the first (low wine) retort. The vapor passing out of the low wine retort is enriched further to about 75% abv. The same process is repeated in the second (high wine) retort. The vapor leaving the high wine retort is about 85% alcohol. After the final strength drops below a fixed value, the still is boiled out to feints. These may be used to charge the retorts for future distillations, and the process repeated.

TWO COLUMN DISTILLATION

A 2-column still is basically the Coffey still. While it does not yield a neutral spirit, it is 'cleaner' than a pot distillation. Its main advantage however is that it can be 'tuned' to give the desired congener profile. This is done by deliberately overloading the rectifying column to varying degrees meaning a greater proportion of the congeners come over in the product. For example a light rum may have a fusel oil draw of 1000 mL/min and a heads draw of 600 mL/min, a medium rum may have a fusel oil draw of mL/min and a heads draw of 400 mL/min and a heavier rum may have a fusel oil draw of 300 mL/min and a heads draw of 200 mL/min. This gives the blender a full pallet to produce the desired blend.

PRODUCTION OF LIGHT (WHITE) RUM

The production of light beverages and hydrofining is discussed in the chapter on beverage distillation. Molasses has some important differences from other feedstocks in that it has very little pectin to break down into methanol. As such, it is generally not necessary to install a demethylizing column. The problems with mud and scaling are covered in the chapter covering molasses as a feedstock. It is important that the wash column is designed to handle mud and scale. Sulfur is often a problem in molasses fermentations. The distillation system must have

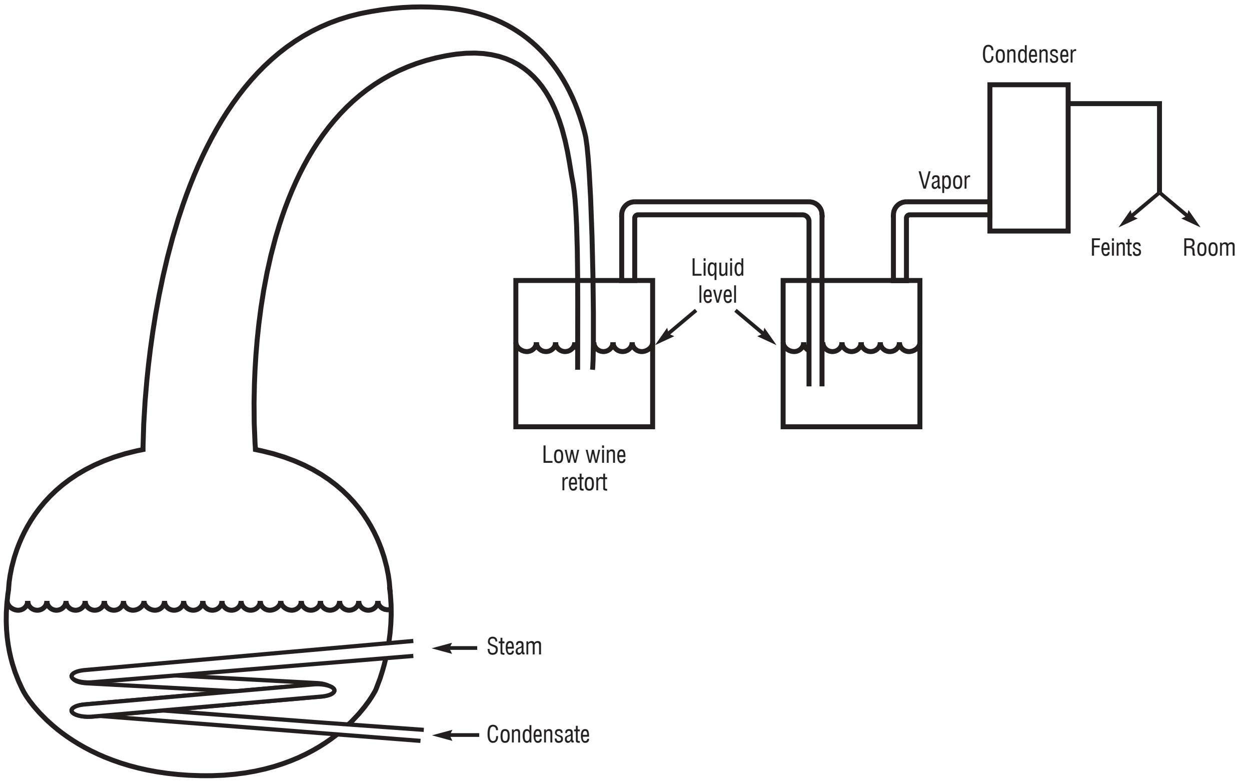


Figure 1. A double retort pot still.

plenty of copper contact. A heads concentrating system such as a barbet head or a separate heads column may be needed in some cases.

Maturation and flavor developement

Esterification is very important in developing the flavor profile of rum. It is a chemical reaction and so is affected by time, temperature and the components present. It takes place at a relatively steady rate during the maturation of rum. (Reazin, 1983) Some esters are also formed during fermentation. It is a reversible reaction between an alcohol and an organic acid. For example:

Ethanol + Acetic acid → Ethyl acetate

MATURATION

Maturation of rum is a complex subject; and the following is intended only as a brief description. As mentioned previously, white rum is generally not wood-aged. Amber and dark rums are aged in wood barrels, most commonly once-used bourbon barrels of American white oak. Some of the barrels are used charred, and others are de-charred before use. Flavor components such as syringic acid, vanillin, syringaldehyde and gallic acid all have their origin in the lignin material of the oak barrels (Lehtonen, 1982). Figure 2 compares the barrel aging components in Scotch, cognac and dark rum. These can be affected by many things including the number of times the barrel has been used, degree of toasting, depth of char and maturation temperature. In some Asian countries local woods are used for the barrels, imparting a unique flavor to the spirit produced in that region. Rum, in contrast to whisky, is generally aged in a warm climate. This changes the maturation process in that some reactions speed up while others progress at the same rate as the cooler-maturing spirits. As can been seen from the graph in Figure 3, the greatest increase in color, tannins,

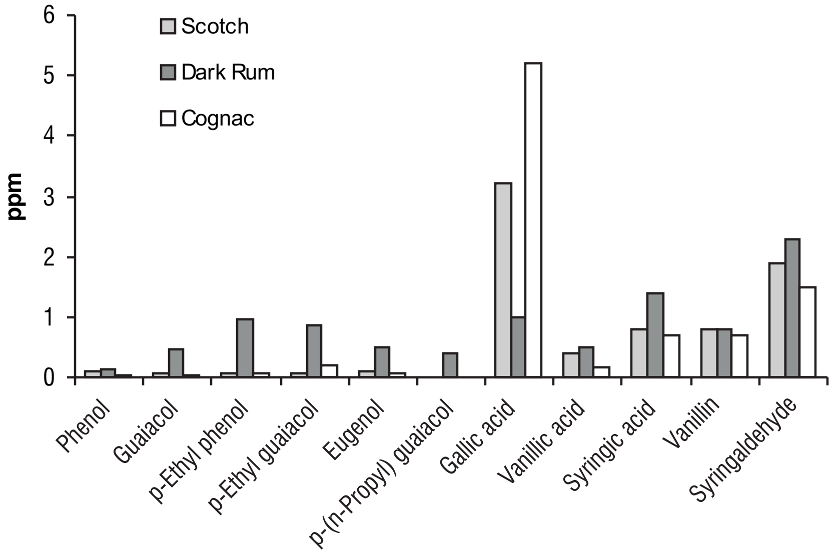


Figure 2. Comparison of aging components in cognac, dark rum and Scotch whisky.

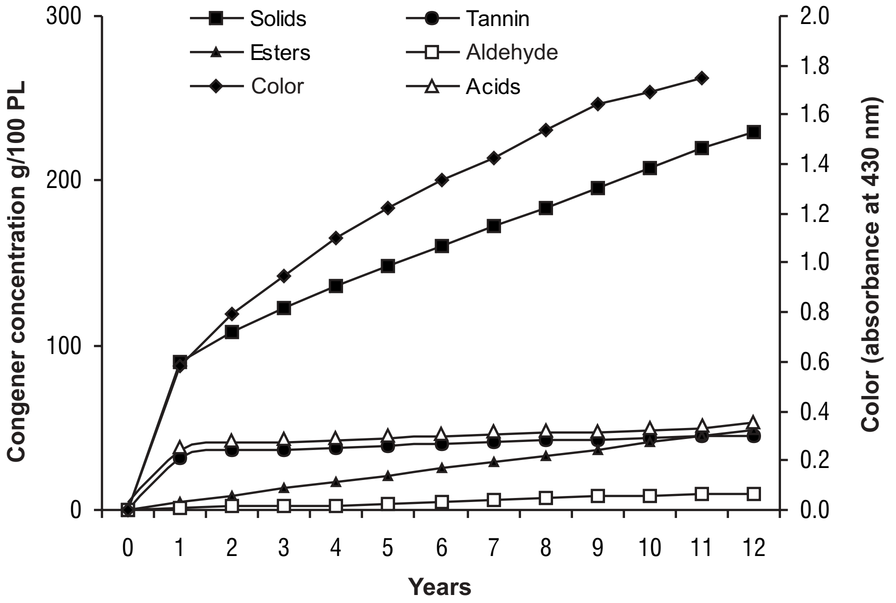


Figure 3. Increase in congeners with aging.

acids and solids occurs in the first year while esters and aldehydes form at a steady rate thought the maturation process (Reazin, 1983).

References

Arroyo, R. 1941. The manufacture of rum. Sugar 36(12).

Arroyo, R. 1942. The manufacture of rum. Sugar 37(1), (5) (7).

Arroyo, R. 1945. The Production of heavy bodied rum. International Sugar J. 11(8).

Arroyo, R. 1948. The flavour of rum - recent chromatographic research. International Sugar J. 50:210.

Lehtonen, M. 1982. The chromatic determination of some organic trace compounds in alcoholic beverages. Presentation, Helsinki.

Lehtonen, M. and Jounlea-Eriksson. 1983. Flavour of distilled beverages (J.R. Piggot, ed). Ellis Horwood Ltd.

Murtagh, J. 1999. Feedstocks, fermentation and distillation for production of heavy and light rums. In: *Alcohol Textbook, 3rd ed.* (K.A. Jacques, T.P. Lyons and D.R. Kelsall, eds), Nottingham University Press, UK.

Reazin, G. 1983. In: Flavour of Distilled Beverages (J.R. Piggot, ed). Ellis Horwood Ltd.

Chapter 17

From pot stills to continuous stills: flavor modification by distillation

ROBERT PIGGOT

Alltech Inc., Nicholasville, Kentucky, USA

Introduction

Since at least the early 8th century men have been increasing the strength of their fermentations. The first attempts were freezing the mash and removing the ice. Later distillation was developed. Distillation is the separation of components by taking advantage of their relative volatilities. When a liquid is boiled its vapor will be richer in its more volatile components. When this vapor is condensed, the resulting liquid will have a higher concentration of the more volatile component. This process can then be repeated.

Pot distillation

Until the advent of practical continuous distillation by Stein in 1827 and Aeneas Coffey in 1830, all distillations were carried out using pot stills. Although simple, pot stills continue to be used to produce the finest Scotch malt whisky, cognac and Irish whiskey. They have evolved from simple small retorts to unique complex pieces of technology (Nicol, 1989). Development took place in Scotland during the nineteenth century as each distiller tried to differentiate himself from the distiller next door. Once set, however, the still design, even to dents, and production methods are not changed (Bathgate, 2003). Pot stills are also used in production of extracted flavor drinks such as gin. While the apparatus is simple it takes skill to produce fine beverages with this type of distillation. The pot still is made of copper. Originally copper may have been chosen because the metal was durable and easy to work and a good conductor of heat. It has proved to be an important part of the distillation process. It is more recently that another attribute, its ability to influence the flavor of the distillate, has been fully appreciated (Nicol, 1989). Copper aids in the removal of undesirable components, especially sulfur, which imparts an off flavor to the distillate.

There are basically two ways to run a pot still. In the first the wash is added to the pot, and then steam is applied to the coils to start the liquid boiling. The collection of the alcohol enhanced condensate is continued until the alcohol remaining in the pot is not economical to remove. To prevent solids burning on the coils, it is important that the liquid is always kept above the steam coils. The spirit derived from this type of distillation is normally very rough as it contains most of the congeners from the fermentation and it is often redistilled to remove some of these congeners. The other way to operate a pot still is to take three fractions from the distillation. The first spirit off, called the foreshot or heads cut, is removed and stored separately. The mid-run is the next spirit off, and it is kept for further distillation. The last spirit off, called the tails cut, contains the heavy fusel

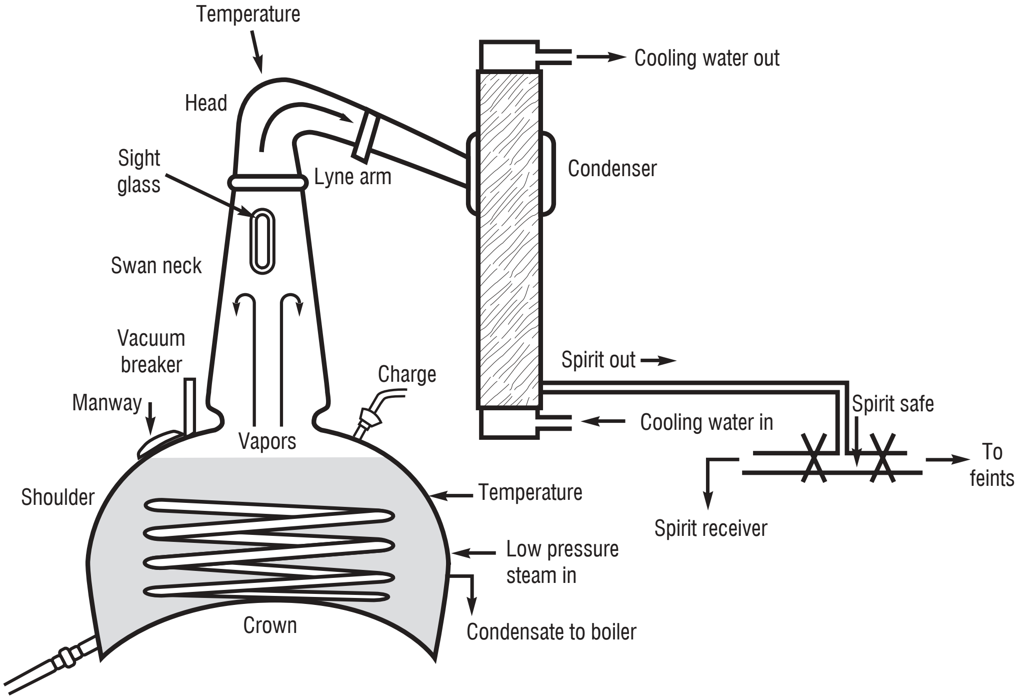

Temperature
Head
Sight glass
Lyne arm
Swan neck
Vacuum breaker
Manway
Vapors
Charge
Shoulder
Temperature
Low pressure steam in
Condensate to boiler
Crown
Cooling water out
Condenser
Spirit out
Cooling water in
Spirit safe
To feints
Spirit receiver

Figure 1. Schematic of a wash still.

oils and is removed at the end of the distillation. Distillation is continued (steam is normally increased at this time to speed the process time) until all practical alcohol has been removed (pot temperature approximately 98°C) the distillation is complete and the spent wash is discharged from the base of the still. The mid-cut from the first distillation, called low wines, (20-25% alcohol) is distilled again in similar fashion. Irish pot distilled whiskey is distilled three times, cognac twice. The result is spirit containing 65-75% alcohol that has the characteristics that the distiller deems right for aging. This sounds like a very simple process, but there are many factors that can influence the characteristics of pot distilled spirit.

WASH

The wash must be from a fermentation that is complete, free from infection and in which the yeast has not been stressed or produced excess fusel oils. The ingredients used to make up the wash will add their own individual characteristics to the final distillate. Water should be soft, clear and free from off odors or tastes. Wine used in brandy production should be as low as possible in sulfur compounds. While it is obvious that different sugar sources, molasses, fruit or grain will produce different spirits, more subtle influences such as fermentation temperature will also affect the final product.

HEADS CUT

The wash strength and the cutoff points for the heads and tails cuts are only changed after very serious consideration as such changes will probably alter the flavor profile of the resulting spirit. The amount of heads removed at the start of distillation will affect the volatile top note of the distillate and remove the ‘low boilers’ (compounds with low boiling points) such as methanol and acetone from the product. The first cut has a role often overlooked, which is to remove the heavy oils and fatty acids in the swan neck, lyne arm and condenser left over from the previous distillation. The historic way to change from the heads cut to the mid-cut in Scotland is to perform a haze test, when it no longer turns

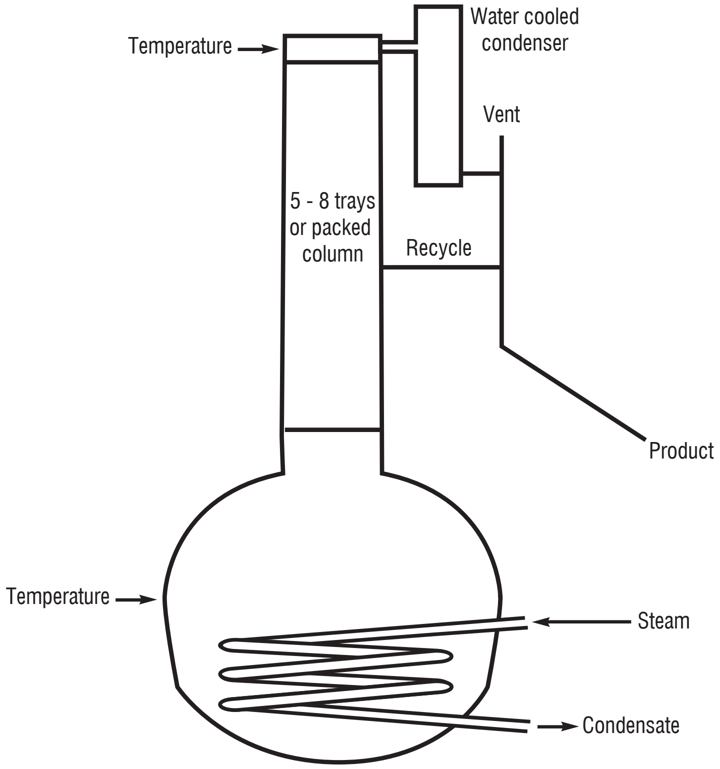


Figure 2. Schematic of a pot still with a distillation head.

cloudy when mixed with water. These days the change is based on time or volume measurement.

TAILS CUT

While some of the heavier alcohols are needed to give the spirit some body, too much results in an oily, unpleasant character and may cause haze problems when diluted to bottling strength.

STILL DESIGN

The design of a still will affect the characteristics of the product. Length and configuration of the swan neck will influence the amount of reflux. Another variation on the pot still is to put a head, either packed or with cross flow trays, in the vapor path (Figure 2). This allows a cleaner, more consistent product. It is popular in small distilleries that cannot afford or justify a continuous still. There are also some very large pot stills used by major beverage alcohol producers for flavor production.

DISTILLATION RATE

The amount of steam applied to a pot distillation will affect the final spirit quality. When the desired final result is obtained, the staging and amount of the steam addition should be kept constant. Too much steam can result in foaming, liquid carryover, and very little internal reflux. A light steam load can give the product a 'stewed' character. The large internal reflux will produce a spirit lighter in character, but will result in more time for chemical combinations to occur during distillation. The resulting energy losses raise the distillation cost.

CONTROL AND AUTOMATION

Traditionally, the changes from heads to mid-cut and then to tails have been accomplished by measuring spirit density after the condenser as it flows through the 'safe'. The safe is a device designed to let the distiller control the process without actually removing a sample. Use of a spirit safe allowed distillation to proceed without the presence of customs and revenue personnel

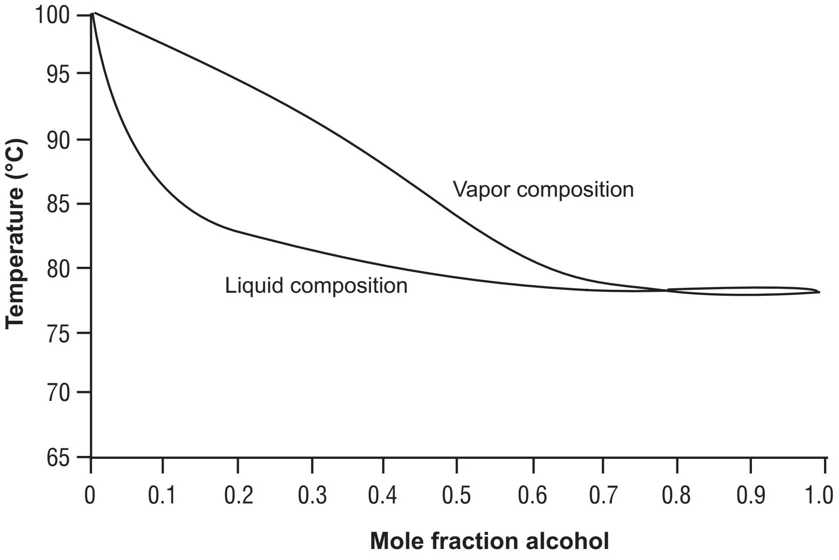


Figure 3. Relationship of temperature and mole fraction of alcohol.

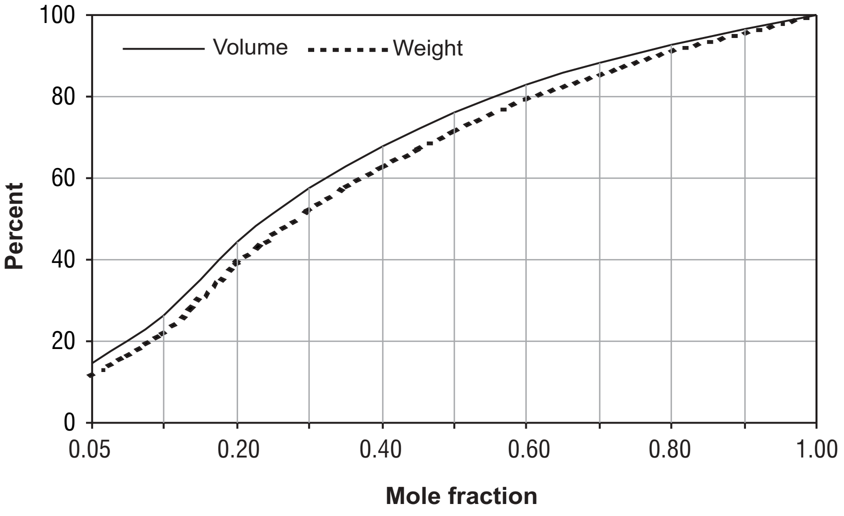


Figure 4. Converting between mole fraction and percent alcohol.

who are very sensitive about the removal of spirit on which the excise duty has not been paid.

Today many pot stills are automated. As can be seen in Figures 3 and 4, if we know the temperature of either the liquid or vapor phase we can know the composition. This is generally the way that pot distillations are automated. Since the feedstock can vary in strength and flow can be erratic at the start of a distillation, it may be better to take the heads cut on a volume basis. At the start of distillation the flow is valved to a container. When a level probe is activated by the distillate, flow switches to the product receiver. A valve opens, dumping the container into the feints tank. When the temperature indicates that the mid-cut is finished, the flow is returned to the feints tank until practically all alcohol has been removed. This temperature is about 98°C. Above that temperature, the steam cost is worth more than the alcohol.

Production of flavored beverages

There are several products which extract flavor by redistillation in a pot still, gin being the most common. While gin may be made by adding flavors to neutral spirit, the best gins are distilled with botanicals to extract the subtle characteristics of these flavored ingredients. The following is an excerpt from the EU regulations on gin:

"The drink may be called 'distilled gin', if it is produced solely by redistilling organoleptically suitable ethyl alcohol of agricultural origin of an appropriate quality, with an initial alcoholic strength of at least 96% vol, in stills traditionally used for gin, in the presence of juniper berries and of other natural botanicals, provided that the juniper taste is predominant. The term 'distilled gin' may also apply to a mixture of the product of such distillation and ethyl alcohol of agricultural origin with the same composition, purity and alcoholic strength. Natural and/or nature-identical flavoring substances and/or flavoring preparations as specified at (a) may also be used to flavor distilled gin. 'London Gin' is a type of distilled gin.

Gin obtained simply by adding essences or flavoring to ethyl alcohol of agricultural origin shall not qualify for the description 'distilled gin'."

DISTILLED GIN PRODUCTION

A measured amount of good quality neutral spirit is added to the pot still; and sufficient water with no odor or taste is added to bring the contents to about 60% alcohol by volume. Botanicals are included in weighed amounts. While juniper berries must be included, other botanicals are also used to enhance the flavor profile. Common botanicals used are coriander seed, cassia bark, citrus peel, angelia root, orris root and fennel seed. There are several ways to extract the flavor from the botanicals during distillation. They may be suspended in muslin bags in the swan neck, spread out on a rack above the alcohol level or placed into the alcohol mixture. A standard pot still distillation is then carried out. Steam is added, and the flavor and aroma of the botanicals is carried with the spirit into the receiver. A heads and tails cut is taken as normal. Usually the cut to tails occurs when the vapor reaches 55% by volume.

Continuous distillation

THE COFFEY STILL

The first continuous still was designed and patented by Robert Stein in 1828. It was installed in the Kirkliston distillery near Edinburgh. It was very complex and not a commercial success. In 1830 Aeneas Coffey, an Irish excise officer, developed the more efficient Patent still in Dublin. This design was simpler, more efficient and well suited for distilling whole grain wort first (Bathgate, 2003). The design took the distilling world by storm and was an immediate success (Table 1, Figure 5) (Robson, 2001). This still had metal trays and wooden walls on the column. A few of these early stills are still functional.

Table 1. Coffey stills in operation 1820-1860.

Year	*England*	*Scotland*	*Ireland*	*Total*
1820	0	1	2	3
1840	4	2	13	19
1860	8	12	8	28

Variations on Coffey's design are still in use in the Caribbean rum and the Scotch whisky industries. In several of the rum plants they will vary the flavor of the final product by adjusting the fusel oil and heads draw by a fixed ratio.

Neutral spirit and brown spirits are two main product types from continuous distillation. Neutral spirit can be made from any feedstock but is usually made from grain or molasses. This spirit has very low odor and taste, and is used for non-aged products (known as white spirits) such as gin and vodka. It may also be used to blend with a highly flavored product and aged in wood barrels. This blended product usually has a lighter flavor than its pot or single column still counterpart.

Brown spirits, so called because they develop a color during maturation in wood barrels, have a much stronger flavor. These spirits are distilled to leave some congeners in the final product. This spirit more resembles pot distillation and gives us bourbon, Armangac, some fine rums and Canadian whisky.

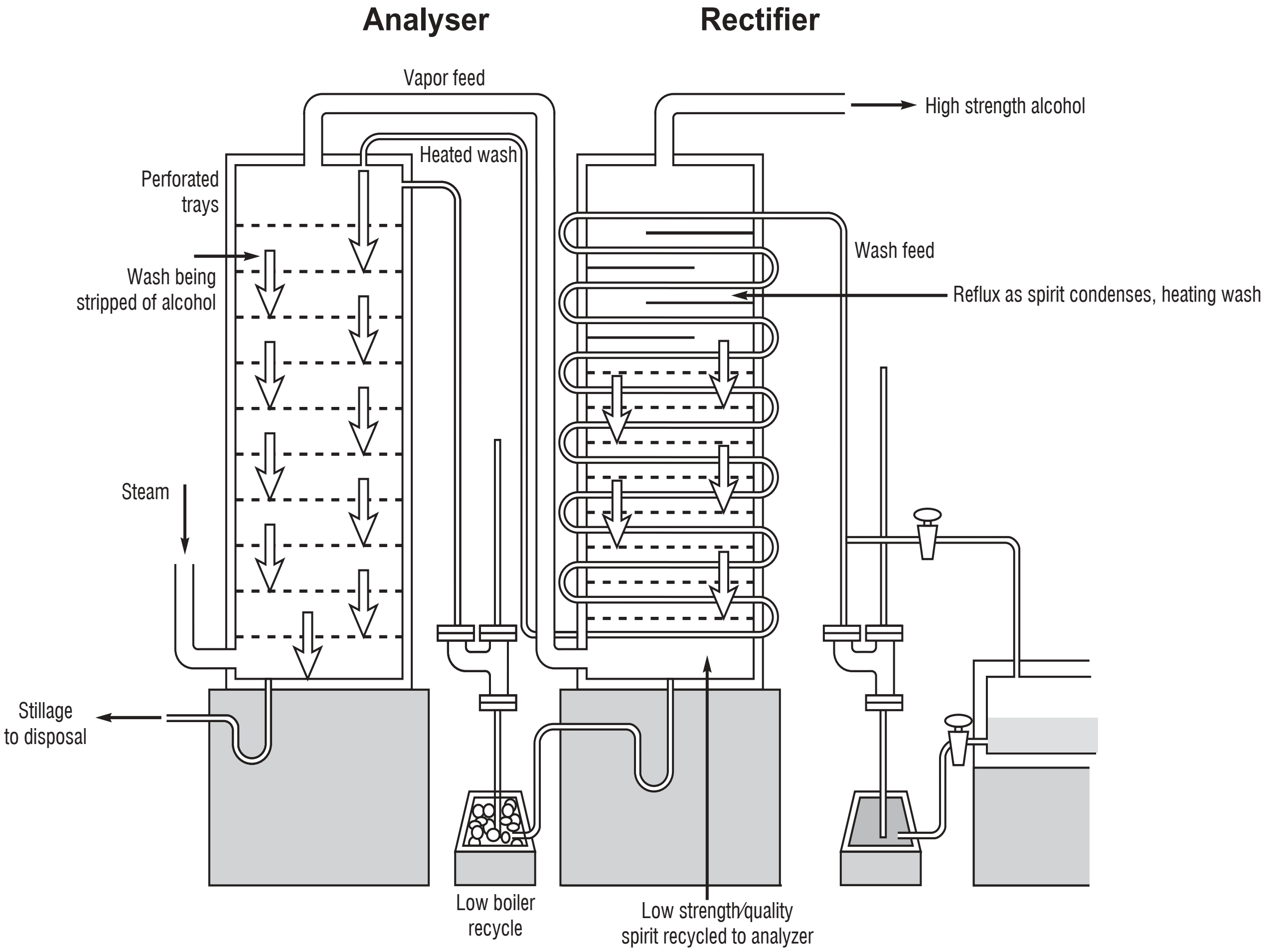

Analyser
Rectifier
Vapor feed
High strength alcohol
Heated wash
Perforated trays
Wash being stripped of alcohol
Wash feed
Reflux as spirit condenses, heating wash
Steam
Stillage to disposal
Low boiler recycle
Low strength/quality spirit recycled to analyzer

Figure 5. Schematic of a Coffey still, circa 1840.

FLAVORED SPIRIT PRODUCTION ON A CONTINUOUS STILL

As with the pot distillation, it is important that the fermented beer or wash is sound. Off odors or taste from poor quality feedstock or contaminated fermentations can carry over into the final product. In the production of Canadian whisky and bourbon, the beer is distilled in a single column that is really a stripping and condensing column stacked together. Some plants use a doubler or thumper (a sort of continuous pot still) after this column.

Operation of a Canadian whisky flavor still

A Canadian whisky flavor still is illustrated in Figure 6. The beer (normally 8 to 10% alcohol by volume, but it can be as high as 15%) is pumped from fermentation though a preheater where it is heated with the overhead vapors from the column. It passes through a tank that vents the liberated carbon dioxide (CO_2) to the atmosphere and then into the column.

Steam enters at the base of the column, either through a reboiler or by direct injection. The column below the feed tray (stripper section) removes the alcohol. The stillage passing out the base should be less than 0.07% alcohol. Since it is a whole mash, it is important that the stripping section uses trays that are not easily fouled by the grain solids or mineral buildup.

The alcohol vapors pass through a solids separation tray into the concentrating section. This section concentrates the alcohol from 55 to 80% by volume. The draw strength has a great effect on the flavor profile of the spirit. Spirit strength is controlled by a temperature reading at the top of the column. This controls either the

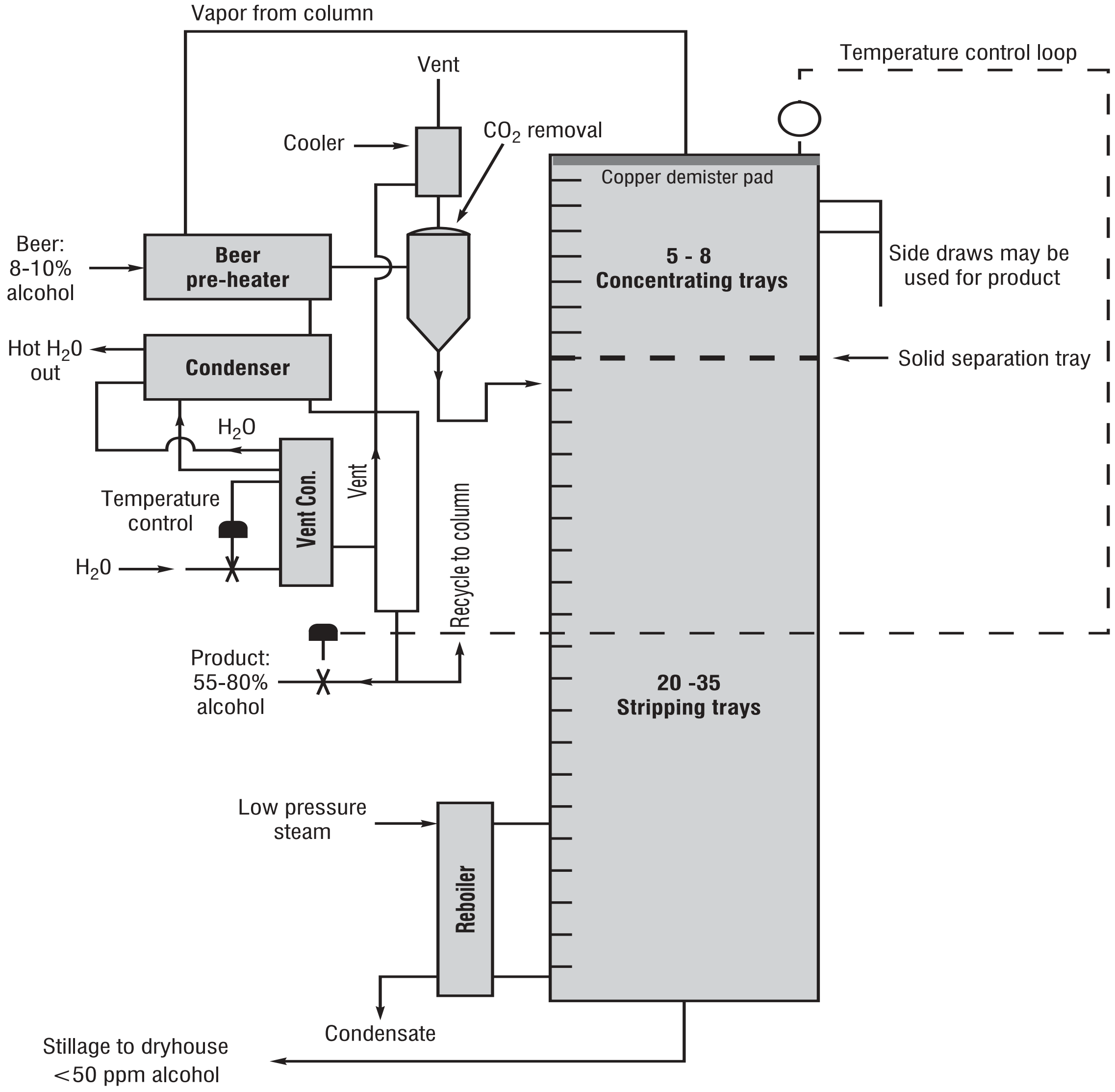

Vapor from column
Vent
Temperature control loop
Cooler
CO_2 removal
Copper demister pad
Beer: 8-10% alcohol
Beer pre-heater
5 - 8 Concentrating trays
Side draws may be used for product
Hot H_2O out
Condenser
Solid separation tray
H_2O
Vent Con.
Vent
Recycle to column
Temperature control
H_2O
Product: 55-80% alcohol
20 -35 Stripping trays
Low pressure steam
Reboiler
Condensate
Stillage to dryhouse <50 ppm alcohol

Figure 6. Typical Canadian whisky flavor still.

amount of reflux or product draw. If temperature is below set point, then spirit is too strong and recycle is reduced. If too high, then spirit is too weak and recycle is increased.

Spirit straight from the still is raw. It is aged for a minimum of three years in small oak barrels.

Production of light and neutral spirit

The product of neutral spirit in a continuous still requires the removal of all components that have odor or taste components over a set threshold. Some products such as light rum require some flavor in the final product, while the specifications for good quality vodka demand no odor or taste. Specifications will also indicate the amount of congeners allowed to be present. These can be less than 1 ppm. Depending on the degree of neutrality required, the rectifying section in a beverage plant can consist of two, three or four columns.

SPIRIT PREPARATION FOR THE RECTIFYING SYSTEM

Many whisky plants in North America also produce a neutral spirit. These plants generally use the flavor column, or one of similar design, as the base alcohol to feed the rectifying system. The strength of this product is usually 70-80%. Due to this low strength, the fusel oils have not been removed. Some heads will be vented or sent to a heads column.

In plants designed to just produce neutral spirit, the spirit is often taken to a higher strength (over 95%). This allows the removal of some fusel oils and heads, similar to the rectification column, before it is sent to the rectifying system. Another possibility is the use of a Barbet head on the column to concentrate and remove the heads (Figure 7).

Extractive distillation or hydrofining

The most common way to remove fusel oils is known as extractive distillation, or hydrofining. The operating principle behind this column is addition of water to the spirit. Ethanol is very soluble in water while the fusel oils are not (Figure 8). The alcohol is added to the column about 2/3 of the way up. The water soluble components (ethanol and methanol) will combine with the water, forming a solution with a higher vapor pressure and hence move down the column. The components that are less water soluble will rise to the top of the column where they are removed. This column requires three components (alcohol feed, steam and water) to be properly balanced. Generally at least eight parts of water for each part of alcohol must be used. In practice much higher ratios (10 to 15) are normally used for good separation.

In order to balance the steam, one must first understand what is happening in the column. The water coming from the top of the column will push the ethanol down the column, while steam coming from the base of the column will try to push it up the column. The result is that the alcohol has a peak in concentration, known as the *pinch point*, about 16 trays from the base of the column. Keeping this pinch in the correct position and strength is a key to the proper balancing of this column. The concentrated fusel oil removed from the top of the column can be sent to a fusel oil washer. Strength at the top of the column will be in the 60 to 68% range. As can be seen in Figure 9, propanol is the most difficult component to remove and is therefore a good indication of how well the column is working. This cleaner low strength spirit is sent to the rectifying column.

Rectification

The rectifying column takes the low strength spirit, removes the last traces of impurities, fusel oils and heads, and takes the strength up to the azeotropic point. One difference in the rectifying column is the large number of trays: over 60 trays is common. This enables a large amount of internal reflux inside the column, which helps concentrate the congeners for better removal (Figure 9). Fusel oils concentrate on the tray where alcohol concentration is 65%; propanol concentrates on the tray with 80% alcohol. It is important that the column is kept stable and that the draws are sufficient to prevent the column becoming loaded with impurities. The cleaner product is removed about five trays from the top of the column. While they concentrate at the top of the column where they are removed, it should be noted that the heads must pass the product draw tray to reach the top of the column. It is therefore not possible to remove all the heads from the product in the rectifying column. To improve alcohol recovery and to increase purity levels, other columns are usually added to the system for demethylization, heads and fusel oil separation. In a well-designed and run extractive distillation system, components other than ethanol will be less than 1 ppm.

FUSEL OIL AND HEADS COLUMNS

These columns take the fusel oil and heads draws from various parts of the process, concentrate them for removal and return cleaned spirit to the process. They are generally small columns with low feed rates that have trays or are packed.

DEMETHYLIZING COLUMN

Certain feedstocks such as fruit and potatoes can end up with very high concentrations of methanol that need to be reduced, or perhaps a product very low in methanol is required. One way to do this, especially if methanol cannot be

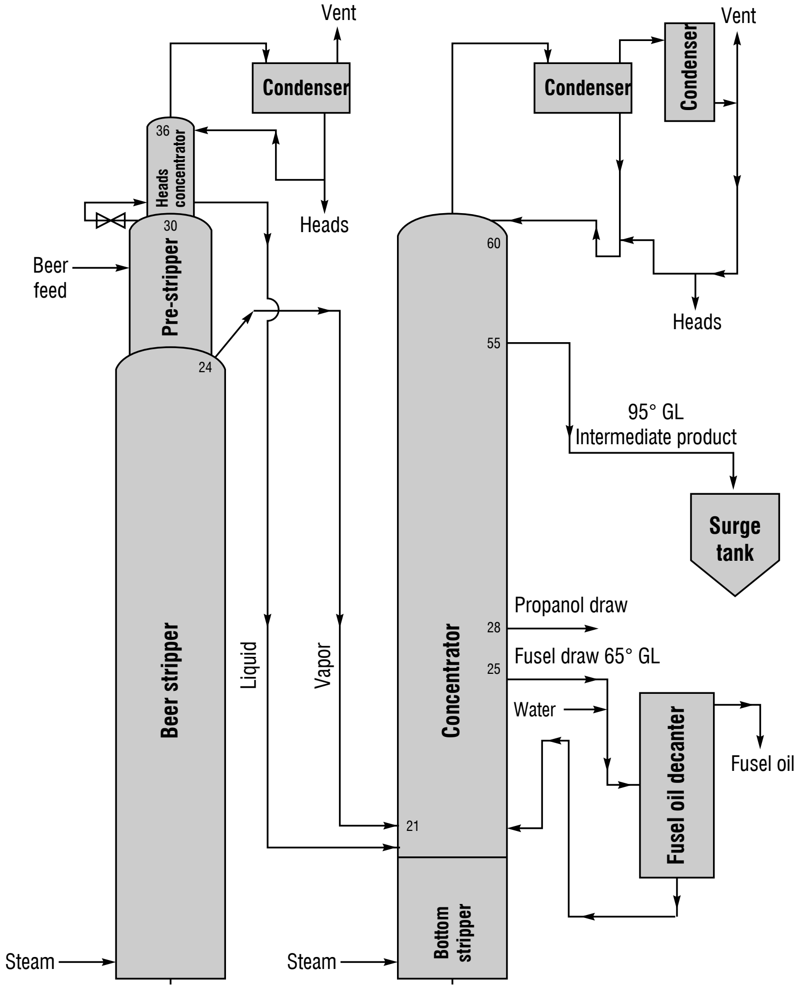

Vent
Condenser
36
Heads concentrator
30
Beer feed
Pre-stripper
24
Heads
Condenser
Condenser
Vent
60
Heads
55
95° GL
Intermediate product
Surge tank
Propanol draw
28
Fusel draw 65° GL
25
Water
Fusel oil decanter
Fusel oil
Beer stripper
Liquid
Vapor
Concentrator
21
Bottom stripper
Steam
Steam

Figure 7. Concentrating neutral spirit: Barbet head on the column to concentrate and remove the heads.

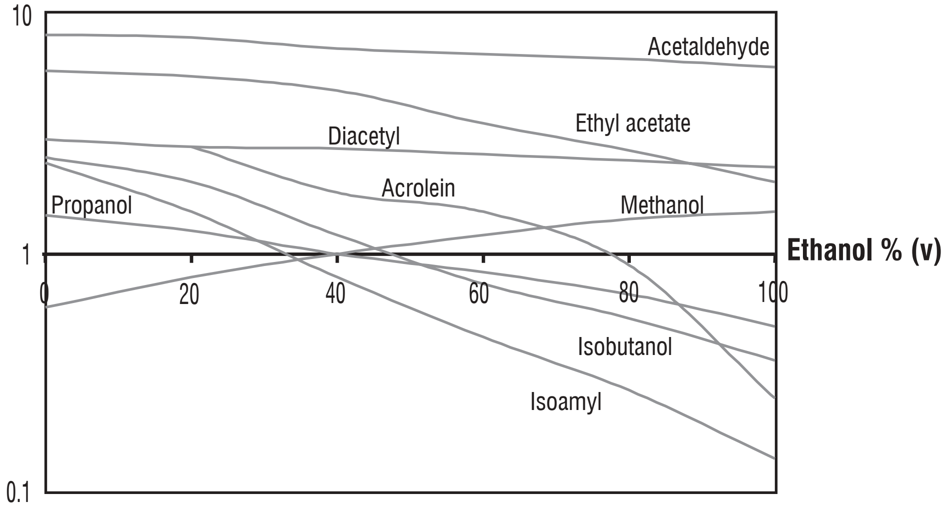


Figure 8. Relative volatility of congeners and ethanol.

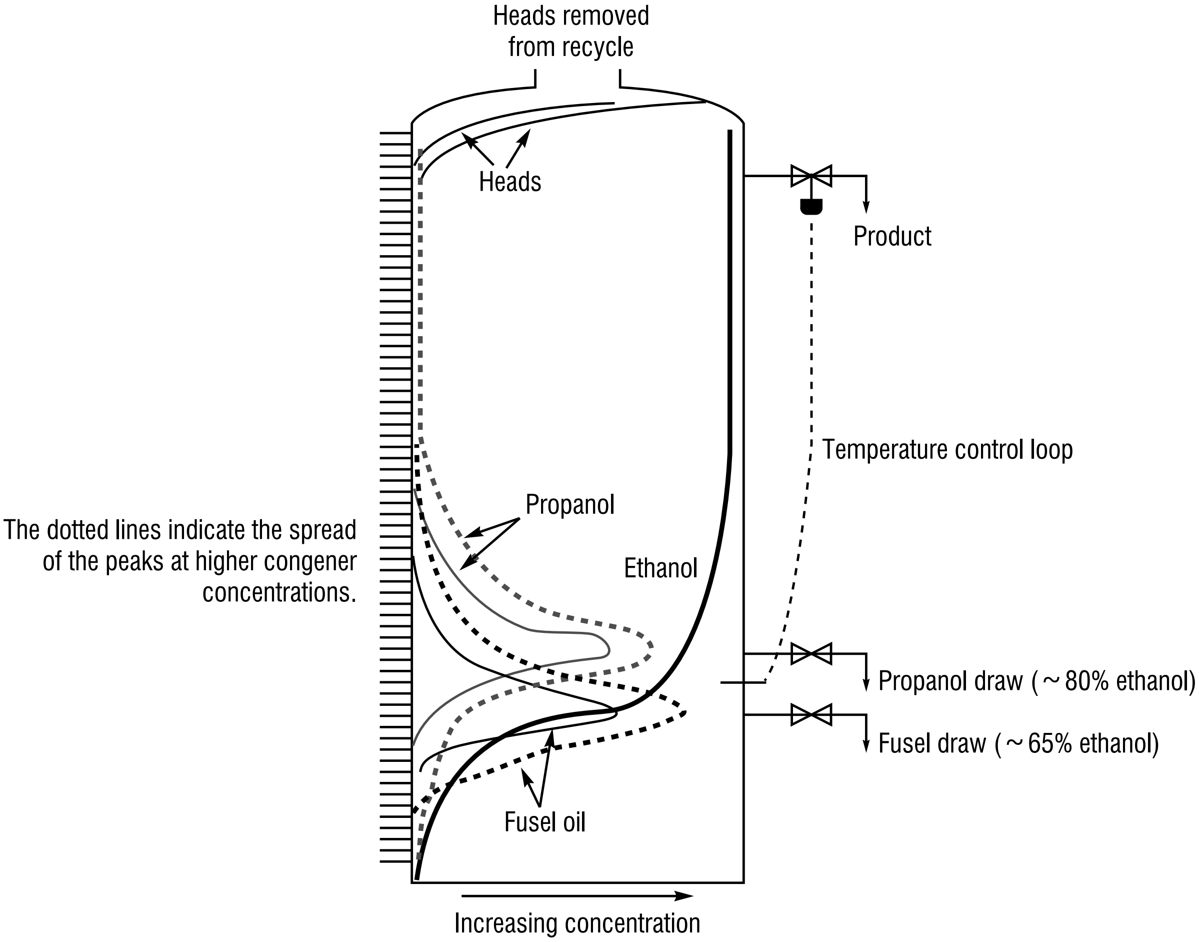

Heads removed from recycle
Heads
Product
Temperature control loop
Propanol
Ethanol
The dotted lines indicate the spread of the peaks at higher congener concentrations.
Propanol draw (~80% ethanol)
Fusel draw (~65% ethanol)
Fusel oil
Increasing concentration

Figure 9. Fusel oil and heads removal in the rectifying column.

removed at the beginning of the process, is to remove it after the rectifying column. Steam to this column is via a reboiler so as not to add water to the system. The alcohol is added to the column about 1/3 to 1/2 of the way up. The lighter methanol and any other heads present are driven up the column and removed. The purified ethanol is removed at the base (Figure 10).

PACKED COLUMNS

Packed columns are known as continuous contact columns. They have an advantage of continuous vapor-liquid contact. Because of this, they may be an advantage when a small column needs an increase in capacity. However, they are only used on clear, non-fouling tasks and as the column size increases they become increasingly complex and not cost effective.

Energy efficiency

Distillation uses a lot of energy, however with good design the energy requirements can be reduced or recovered for other operations.

REBOILERS

Reboilers are simply heat exchangers that take a hot stream and use it to generate steam for another part of the process. This is illustrated in the flavor column (Figure 10). The advantage of this is that the condensate can be returned to

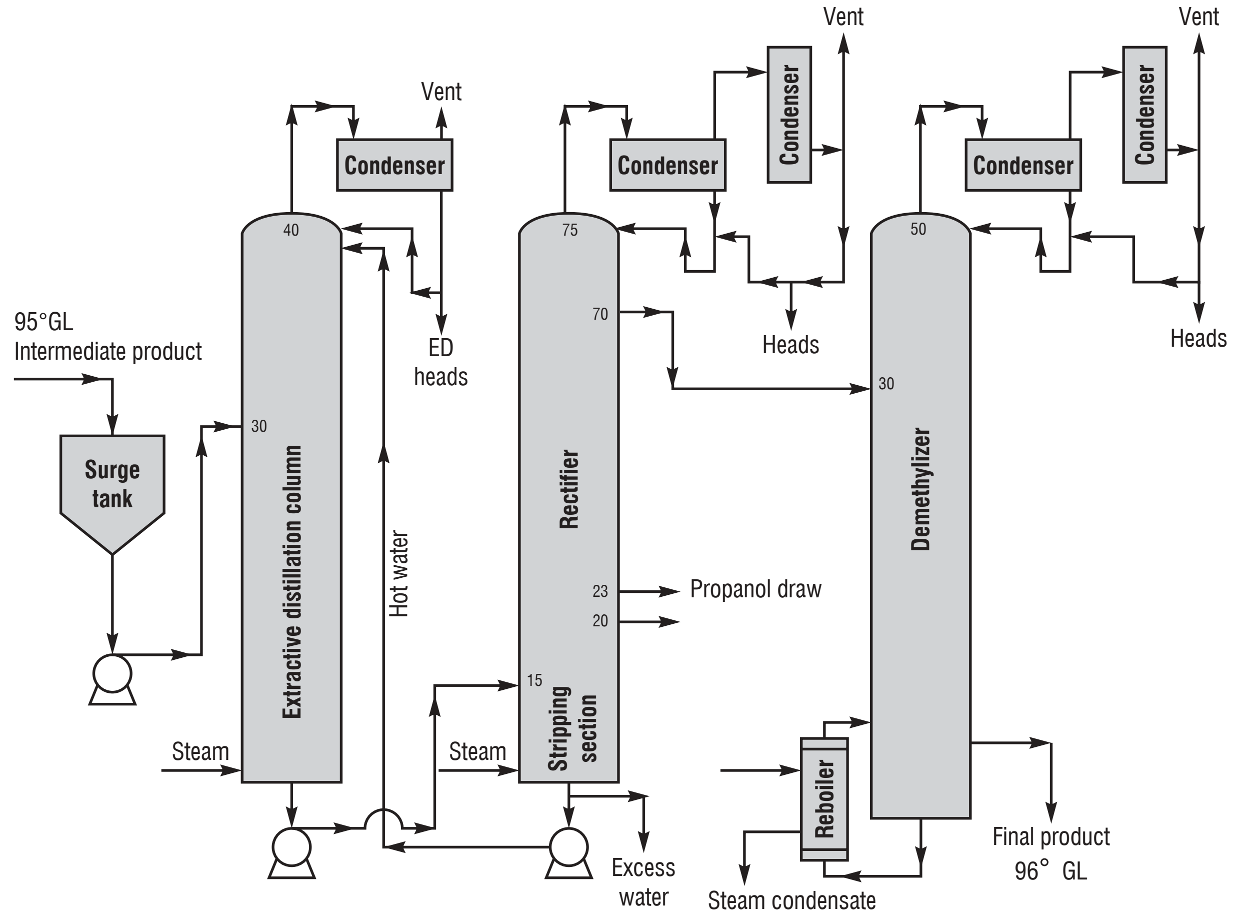

Vent
Vent
Vent
Condenser
Condenser
Condenser
Condenser
Condenser
40
75
50
70
30
30
95°GL
Intermediate product
Surge tank
ED heads
Heads
Heads
Extractive distillation column
Hot water
Rectifier
Demethylizer
23
20
15
Propanol draw
Stripping section
Steam
Steam
Reboiler
Excess water
Steam condensate
Final product 96° GL

Figure 10. Three column rectification system used in neutral spirit production.

the boiler instead of creating extra liquid that must be disposed of in the stillage.

PRESSURE DISTILLATION

One or more columns are run under pressure. Instead of the heads being condensed by cooling water, they are passed through a reboiler that produces steam for another column. This reduces the total distillation steam required.

VACUUM DISTILLATION

In this configuration, one column (usually the stripping column) is run under vacuum. This means heads from an atmospheric column can power the reboiler on the vacuum column. This is becoming common in distilleries using molasses as a feedstock. The lower temperatures in the stripping section reduce calcium fouling of the trays.

THERMOCOMPRESSORS

Thermocompressors are simple, low maintenance devices that can save a considerable amount of energy. High pressure steam is passed through a venturi-type nozzle. This draws a vacuum in the vessel containing the hot liquid causing it to boil. This steam combines with the now-low-pressure steam for use. About 25% (depending on design) of the steam out of the thermocompressor is recovered heat from the liquid. Mechanical recompression is another possible option, but it is costly to install and run so the savings must make it cost effective.

HEAT RECOVERY

The condensers should be viewed as a source of heat. This could be used for feed preheating, heating boiler water feed or any other process that requires hot water such as cooking or CIP systems.

INSULATION

The heat savings obtained by simply insulating columns and other hot vessels to retain heat is often overlooked, but usually gives an excellent return on investment.

Quality assurance

In order to be sure the correct quality product reaches the receiver, a system of predictive quality control must be implemented. This can include such things as a check on the fermentor for odor and taste before it is pumped to the still. Samples of the fusel oil and propanol draws on the rectifying column can be obtained to see if there is a buildup before it gets into the final product. Whatever works in a particular system, the trick is to detect potential problems before they become full-blown problems that affect final spirit quality.

While many countries including the EU have specifications for neutral spirit (the US does not) the companies using the spirit usually have their own much tighter specifications that must be met by the producer.

In these days of large corporations and improved technology there is an increasing trend to use instruments, especially the gas chromatograph, to set standards for beverage alcohol. While these procedures can be of great use in doing predictive testing of the distillation system, they cannot duplicate the human senses when it comes to assessing a product. To do this, it is important that a distillery has a trained staff, proper facilities and procedures for organoleptic testing. Remember that the final customer will not have a gas chromatograph on the bar, but he or she will have a nose and taste buds. The customer is the final test of product quality.

References

Bathgate, G. 2003. History of the development of whisky distillation. In: *Whiskey Technology, Production and Marketing* (I. Russell, ed). Academic Press.

Murtagh, J. Feedstocks, fermentation and distillation for production of heavy and light rums. In: *Alcohol Textbook, 3rd ed.* (K.A. Jacques, T.P. Lyons and D.R. Kelsall, eds), Nottingham University Press, UK.

Nicol, D. 1989. Batch distillation. In: *The Science and Technology of Whiskies*, Longman Scientific and Technical.

Chapter 18

From liqueurs to ‘malternatives’: the art of flavoring and compounding alcohol

ANDY HEAD AND BECKY TIMMONS

North American Biosciences Center, Alltech Inc., Nicholasville, Kentucky, USA

Introduction

Using flavor in alcoholic beverages expands the variety of consistent, high quality products available to the consumer. From high proof spirits that have a twist of fruit flavor or spice, to medium strength cordials, to low alcohol premixed cocktails and ‘malternatives’, the inclusion of flavors gives the beverage producer limitless flexibility in creating products to satisfy the constantly changing beverage alcohol market.

Historically, flavors were added to alcohol to cover up poor quality and bad taste. Gin was first used for medicinal purposes in the 17th century. Juniper berries were added prior to distillation to improve the taste. It was common practice to use poor quality corn and barley and to add a flavor to make the product more palatable. In the 18th century, production of flavored vodkas became popular. This was done to improve marketability of poor quality spirits.

Today, flavors are often added to give a marketing edge to a competitive industry. According to Adams Handbook Advance, “The US distilled spirits industry was up for the 5th consecutive year in 2002”. The number of flavored alternatives is credited with this increase.

Flavor perception

Our tongues sense the four basic tastes of sweetness, sourness, saltiness and bitterness. Additionally our mouths detect textures referred to as ‘mouthfeel’. In beverages these include viscosity and slickness often described as oiliness. Other sensations are drying and trigeminal sensations or salivation stimulation. Some tastes, especially bitterness, tend to linger or are perceived less quickly.

Most of what we describe as flavor perception is actually aroma. Aroma perception occurs in the nose and can occur without tasting. Thousands of aromas are distinguishable. In order for a chemical to be smelled, it must become airborne so that it can reach the air in the nose and be detected. Holding a beverage in the mouth warms it and more of the aromatic compounds are released. Most aroma chemicals are not soluble in water. Alcoholic beverages have an advantage in that rich, intense flavors are produced because alcohol is a natural solvent that is exceptionally volatile. The aroma compounds release more readily from an alcoholic beverage than from a soft drink.

Aesthetics of flavored beverages

Because people ‘drink with their eyes’, the appearance of these lower proof products is important. Approved artificial colors and caramel can produce virtually any shade of color. Adding a homogenized clouding flavor can simulate the inclusion of juice. The complexity of the interaction of these taste, smell and visual sensations is what provides nuance and character to a beverage.

Types of flavored beverages

HIGH PROOF SPIRITS

Although originally considered an inferior substitute for the distiller's art or a mask for product deficiencies, flavor-added spirits are now considered to be the equal of unflavored spirit. As previously mentioned, one of the earliest examples of added flavor in high proof spirits was improvement of inferior spirit flavor by adding juniper berries during distillation. These were the earliest gins. Now, many gin products are made by adding flavor from the refined oil of juniper berries, along with other essential oils, directly to high proof neutral spirits. Refined essential oils are much more consistent than raw botanicals, which can be affected by varying growing conditions.

Blended American and Canadian whiskies use flavors to provide a fuller, more balanced flavor profile while reducing the ingredient costs of more expensive spirits. American whisky blends are made by combining a straight bourbon whisky with neutral alcohol. A well-designed flavor can mimic the flavor compounds lost from the neutral spirit. Vanilla can be added to replace that which would have been extracted from the oak barrel. Higher alcohols and other congeners can be added back in amounts and proportions that are more desirable than the original product. Oak flavor from oak wood is not permitted for flavoring American blended whiskies.

Producers in the crowded vodka and rum market have found that flavor and a little sugar can add an 'extra' that sets a product apart. Citrus extracts and pepper or spice flavors are quite popular in flavored vodkas, which have found a new niche in trendy martini cocktails. Spiced rums, which are high in vanilla flavor, and tropical rums, reminiscent of piña coladas, have also become the base of many popular drinks.

CORDIALS AND LIQUEURS

Cordials, and other medium strength sweetened beverages, are an extremely important segment of the alcoholic beverage market. Coffee, chocolate, vanilla, almond, cherry, peach, orange, cinnamon, hazelnut, anise and even gold leaf are just the beginnings of a list for popular flavors used in this broad category. Sweetened to between 12 and 40% sugar, these products can be consumed by themselves, but are frequently used as ingredients in cocktails, in cooking or even as toppings for deserts.

PREMIX COCKTAILS

Shelf-stable cream-type beverages can be produced using non-dairy cream substitutes. This allows for a whole array of milkshake type products, with names such as mudslides, creamsicles and grasshoppers. Margaritas and similar premixed cocktails give the consumer the convenience of a complete spirits drink without the inconvenience of buying multiple ingredients and the hassle of complex and time consuming preparation.

MALTERNATIVES

'Malternatives' and 'Alcopops' are beverages that contain alcohol at the same level as beer. Malternatives, which are produced in the US, use as a base a malt product with an intentionally low flavor. Flavor and sweetener are added to produce a drink similar to a cocktail from a distilled spirit. Many are marketed using the brand name of a spirit product, e.g. SMIRNOFF ICE®, BACARDI SILVER™, etc. This category has been the most successful of recently introduced product lines. Among the marketing advantages these products have is unlimited flexibility for product design. Virtually any type of cocktail can be developed in this base product. These products can be sold at the same retail outlets as standard beer products, which is a tremendous advantage over spirit-based beverages. Crossover advertising can benefit the spirit product, as well. Distilled spirits have traditionally not been advertised on television in the US, however malternatives with the same brand name can be advertised (e.g. Schmirnoff Ice). The production costs are much lower because of the differential between beer ($18.00 per 31 gallon barrel regardless of alcohol level), wine and flavoring ($2.00 per gallon of alcohol) and distilled spirits excise taxes ($27.00 per gallon).

Alcopops are similar products that are actually made with spirits set to a comparable alcohol level. Smirnoff Ice sold outside the US will contain vodka as the alcohol source in place of a malt base.

Types of flavors

ARTIFICIAL *VS* VATURAL

For the purposes of the Codex Alimentarius *"Natural flavors and 'natural flavoring substances' are preparations and single substances, respectively, acceptable for human consumption, obtained exclusively by physical processes from vegetable and sometimes animal raw materials either in their natural state or as traditionally processed for human consumption."* 'Natural' does not mean coming from the plant that characteristically produces the flavor. The classic example is benzaldehyde derived from peach pits and used in almond or cherry flavors. 'Natural' does not mean more healthful, safer or higher in quality than artificial. It is merely a description of the source.

'Nature–identical flavoring substances' are *"substances chemically isolated from aromatic raw materials or obtained synthetically. They are chemically identical to substances present in natural products intended for human consumption, either processed or not, as defined above."*

Another group of ingredients called 'nature-identical' is comprised of *"substances that are chemically identical to flavoring substances naturally present in vegetable or animal raw materials that are not normally considered as human food."*

The same ingredient can be available in both natural and nature-identical forms (i.e. natural vanilla versus vanilla that is nature-identical). The natural version is typically considerably more expensive and less concentrated. However, there can be valuable reasons for selecting the natural form for a beverage flavor.

'Artificial flavoring substances' are *"those substances which have not yet been identified in natural products intended for human consumption, either processed or not."*

In addition to these classifications the US Bureau of Alcohol, Tobacco and Firearms (BATF) has placed restrictions on the inclusion and labeling of certain ingredients. Artificial flavor substances can be included in an alcoholic beverage product without being labeled artificially flavored if the BATF-approved flavor contains 0.1% or less of an artificial ingredient. There are also restrictions on the level of certain ingredients, whether or not they are artificial.

KOSHER

Foods, beverages, raw materials and processing aids are kosher if they are produced in accordance with the Jewish dietary laws. There are several rabbinical services that will, for a fee, certify processes, equipment and ingredients as kosher and allow their seal to be placed on product packaging. Although these certifications are useful for marketing purposes, Kosher does not mean more healthful, safer or superior in quality to non-Kosher.

BOTANICALS

These are the starting point for most natural flavors. Botanicals are parts of a plant that contain aromatic chemicals that are desirable as flavoring agents. All parts of plants have been used for flavor. A few examples are juniper berries, hop flowers, leaves and stems, rose petals and hips, cinnamon leaf and bark, whole peppers and pepper corns, clove buds, angelica root and citrus rinds. Non-plant derived natural flavoring agents include beeswax, castoreum and fermentation congeners.

EXTRACTS

It is frequently useful to remove the aromatic compounds from the whole plant material prior to use. Essential oils, absolutes and concretes are the fragrant chemicals found in the plant material. There are various extraction techniques. Expressed oils are squeezed out in presses. Carbon dioxide, propylene glycol, steam and ethanol are commonly used for extraction of essential oils. Further purification can be achieved by distillation into various fractions based on boiling points.

Flavor delivery systems

Most aroma chemicals are hydrophobic, not soluble in water. In order to use them in a beverage they must be made soluble. The three commonly used delivery systems for putting hydrophobic flavors into beverages are solutions, emulsions and extracts. Solutions are simple mixtures made by dissolving flavor ingredients in solvents that can be mixed into water. Typical solvents are ethanol and propylene glycol. Extracts or hydro-alcoholic extracts take advantage of the different solubility limits of the different compounds in essential oils, especially citrus oils. Citrus extracts are made by dissolving a citrus oil or blend of oils in high proof alcohol and then combining or 'shocking' with water. The less soluble compounds will come out of solution and rise to the top and can be decanted. Fortunately, with citrus oils the compounds that are soluble in the alcohol/water portion are the more organoleptically pleasing compounds. The insoluble terpenes, which have been removed, are typically astringent and bitter. The remaining extracted portion is water-soluble.

Emulsions are made by homogenizing the flavor ingredients in a solution of a gum. A gum is a high molecular weight carbohydrate polymer. The oil droplets are trapped in the gum matrix and will not re-coalesce when mixed into the beverage. Another use of emulsions is as a clouding agent. If a clouded product is desired, such as to simulate limejuice in a margarita premix, a homogenized ester gum flavor can produce the desired effect. Clouding agents can also be used to add variation to a product line, such as with Gatorade's Frost line of products.

Tax benefits of flavored beverages in the US

The alcohol portion of a flavored beverage is usually provided by neutral spirits, but whisky or other spirit can be used. Alcohol used in the flavor contributes to the total alcohol as well. Many of these products take advantage of the so-called 'wine option'. This is a tax savings available by using a neutral wine, which can be included at up to 49% of the alcohol in the product after the alcohol from the flavor is taken into account (example formulations in Tables 1 and 2).

A significant, but frequently overlooked, method of cost reduction available in the distilled spirits industry is the use of flavors to reduce the level of taxable alcohol. Alcohol used in flavors is taxed at a substantially lower rate than distilled alcohol used in beverages. When a flavor is added to a spirits beverage, the alcohol it contains is included in the total proof. However, alcohol contained in the added flavor, when kept under 2.5% of the total proof of the finished beverage, is not taxed as beverage spirits alcohol. Because the tax burden on beverage spirits is so high relative to non-beverage alcohol ($13.50 per proof gallon or $27.00 per gallon of pure alcohol for beverage distilled spirits as of this writing), it is possible to add a flavor that costs less than the tax. Flavor producers are able to formulate alcohol-containing flavors that will not alter the flavor profile of the finished product, thus allowing the full savings available from this tax benefit. Flavoring substances must be compounds judged by BATF to be 'unfit' for human consumption at full strength, but can be developed such that when diluted and/or combined with other flavors, the desired flavor profile is achieved. A judicious use of flavors containing alcohol can reduce costs in alcoholic beverages containing distilled spirits (See Tables 3-5).

Building a beverage

Creating a new alcoholic beverage requires decisions at every step. One first must decide on the alcohol level and source. In addition to beverage type, tax rates and marketing regulations affect this decision. For example, there might be a marketing benefit to using rum to make a premixed cocktail, but the cost might be prohibitive. A combination of rum and neutral spirits might be the compromise. Malt and other than standard wine can only be used in products that are relatively low in alcohol level, i.e. cordials and malternatives. They also have characteristic flavor and so must be used in heavily flavored products.

Next, the flavor is selected. This is typically the bottleneck of the project. Choosing the perfect flavor, or combinations of flavors to give

Table 1. Vodka with full 'drawback'[1].

	Vodka @ 80 proof (full drawback)		*Vodka @80 proof*	
	Wine gallon	*Proof gallon*	*Wine gallon*	*Proof gallon*
Vodka (@ 190 proof)	41.05263	78	42.10526	80
Neutral blender (@180 proof)	1.11111	2	0.0	0
Water	95	0	95	0
Total	100.00	80	100.00	80
Taxes				
Tax paid at $13.50/proof gallon for distilled spirits	$1053.00		$1080.00	
Tax paid at $1.00/proof gallon for approved neutral blender	$1.11		$0.00	
Total	$1054.11		$1080.00	

Tax difference = $1080.00-$1054.11 = $25.89/100 wine gallons (finished goods)

One case of 12 x 750 ml bottles = 2.38 wine gallons

2.38 wine gallons/case x $0.2589 tax reduction/wine gallon = $0.61 tax savings per case.

[1] Full drawback is the term describing the maximum advantage taken of the lack of excise tax on alcohol in flavors (i.e. 2.5% of total alcohol). In a flavored 80 proof vodka, 2 proof degrees or 1/40th of the alcohol is non-taxable.

Table 2. Margarita premixed cocktails.

	25 proof		*25 proof with wine option and drawback*	
	Wine gallons	*Proof gallons*	*Wine gallons*	*Proof gallons*
High proof tequila (@ 150 proof)	16.63	24.945	8.287	12.431
O.T.S. wine @21% aav[2]			28.428	11.944
High fructose corn syrup (72°Brix)	15.00		15.00	
Neutral blender @ 180 proof			0.317	0.57
Orange Extract™[1] @50% aav	0.01	0.005	0.01	0.005
Lime Extract™[1] @ 50% aav	0.1	0.05	0.1	0.05
Neutral Cloud Flavor™[1]	0.05		0.05	
Citric acid, lbs	4.2		4.2	
FD&C Yellow 5, lbs	0.1		0.1	
FD&C Yellow 6, lbs	0.02		0.2	
Water	QS		QS	
Total	100	25	100.0	25

[1] Alltech Inc.

[2] Absolute alcohol by volume

the intended effect, can take time. Any changes in the other parameters can put you back to the drawing board with the flavor.

Sweetness level is based on the beverage type and flavor. As little as 1% sugar can smooth the roughness of a high proof spirit. Premixes blended with ice need the sugar level sufficiently high to prevent the product becoming watered down after dilution. Fruit flavored beverages may have different sweetness levels depending on the fruit flavor. Sugar also adds viscosity to a beverage. Some products such as eggnogs can be made more viscous by adding gums such as xanthan.

Acids (usually citric and sometimes with sodium citrate) are used in most cocktails to provide tartness and simulate juice content. The correct acid/sugar ratio is important for giving the right taste and mouth feel to a drink. Coffee and vanilla beverages require little or no acid addition. Cream drinks are incompatible with acid, so the flavor selected for these products should not need tartness to enhance flavor. Malt, which is fermented in an acidic pH range, is also not suitable for cream drinks.

After the flavor profile is completed the aesthetic issues of color and cloud are addressed. Many citrus flavored drinks are clouded to simulate juice content. Others such as a melon cordial would be shiny with only some color added. Deep color suggests richness and enhances the flavor experience.

Table 3. Irish cream cordial with full drawback @ 50 proof.

	Wine gallon	*Proof gallon*
Irish whiskey @ 65% aav[1]	37.5	48.75
Neutral blender™[2] @ 90% aav	1.389	1.25
High fructose corn syrup @72°brix	12.0	
Non-dairy cream substitute, lbs	200	
Water (homogenize with cream substitute)	25.00	
Non-alcoholic vanilla flavor™[2]	0.1	
Caramel color, wine gallons	0.01	
Water	QS	
Total	100	50

[1] Absolute alcohol by volume

[2] Alltech Inc.

Table 4. Strawberry malternative @ 6% a.a.v.

	Wine gallon	*Proof gallons*
Beer @ 10% aav[1]	30.0	6.0
Strawberry Flavor™[2] @ 60% aav	5.0	6.0
High fructose corn syrup @ 72°brix	12.0	
Citric acid, lbs	2.5	
FD&C Red 40, lbs	0.5	
Neutral Cloud Flavor™[2]	0.1	
Water	QS	
Carbonate to 3.5 volumes	100.0	12.0

[1] Absolute alcohol by volume

[2] Alltech Inc.

Table 5. Conversion tables for computation of taxable quantity of spirits.

Bottle size	*Equivalent fluid ounces*	*Bottles per case*	*Liters per case*	*US gallons per case*	*Corresponds to*
1.75 L	59.2	6	10.50	2.773806	1/2 gallon
1.00 L	33.8	12	12.00	3.170064	1 quart
750 mL	25.4	12	9.00	2.377548	4/5 quart
375 mL	12.7	24	9.00	2.377548	4/5 pint
200 mL	6.8	48	9.60	2.536051	1/2 pint
100 mL	3.4	60	6.00	1.585032	1/4 pint
50 mL	1.7	120	6.00	1.585032	1, 1.6 & 2 oz

Official conversion factor: 1 Li = 0.264172 US gallon
TTB, 2003

Table 6. Conversion tables for computation of taxable quantity of wine.

Bottle size	*Equivalent fluid ounces*	*Bottles per case*	*Liters per case*	*US gallons per case*	*Corresponds to*
3.0 L	101	4	12.00	3.17004	4/5 gallon
1.5 L	50.7	6	9.00	2.37753	2/5 gallon
1.0 L	33.8	12	12.00	3.17004	1 quart
750 mL	25.4	12	9.00	2.37753	4/5 quart
500 mL	16.9	24	12.00	3.17004	1 pint
375 mL	12.7	24	9.00	2.37753	4/5 pint
187 mL	6.3	48	8.976	2.37119	2/5 pint
100 mL	3.4	60	6.00	1.58502	2, 3 & 4 oz
50 mL	1.7	120	6.00	1.585032	1, 1.6 & 2 oz

Official conversion factor: 1 L = 0.264172 US Gallon
TTB, 2003

Table 7. Tax and fee rates.

Product	*Tax*	*Tax per package (usually to nearest cent)*
Beer	*Barrel (31 gallons)*	*12 oz. can*
Regular rate	$18	$0.05
Reduced rate	$7 on first 60,000 barrels for brewer who produces less than 2 million barrels	$0.02
Wine	*Wine gallon*	*750 ml bottle*
14% & under	$1.07[1]	$0.21
Over 14 to 21%	$1.57[1]	$0.31
Over 21 to 24%	$3.15[1]	$0.62
Naturally sparkling	$3.40	$0.67
Artificially carbonated	$3.30[1]	$0.65
Hard cider	$0.226[1]	$0.04

[1] $0.90 credit, or for hard cider $0.056, for first 100,000 gallons removed by a small winery producing not more than 150,000 wine gallons per year. Decreasing credit rates for winery producing up to 250,000 wine gallons per year.

Distilled spirits	*Proof gallon*	*750 ml bottle*
All	$13.50 less any credit for wine and flavor content.	$2.14 (at 80 proof)

This was last updated on November 23, 1999 (www.ttb.gov)

Conclusions

The beverage alcohol industry is increasingly competitive. Producers are in a continuous struggle to increase profits and market share and consumers are looking for the latest trend and more convenience. The art and practice of flavoring beverage alcohol provides short product development times for a wide variety of products, improves consistency and quality of products and can reduce tax liability and improve profits.

References

Code of regulations- *http://www.ttb.gov/regulations/27cfr5.html*

TTB. 2003. Tobacco Tax and Trade Bureau, BATF, Dept. of the Treasury. *www.ttb.gov.*

2003 Adams Handbook Advance, Adams Beverage Group, Norwalk, CT

Chapter 19

Production of American whiskies: bourbon, corn, rye and Tennessee

RON RALPH

Ron Ralph & Associates Inc., Louisville, Kentucky, USA

Introduction: definitions of bourbon, corn, rye, wheat, and Tennessee whiskies

The US Bureau of Alcohol, Tobacco and Firearms (BATF) has set specific guidelines to define all types of alcoholic beverages produced in the United States. The general definition of whisky is 'a spirit aged in wood, obtained from the distillation of a fermented mash of grain'. This spirit can be produced from any grain or combination of grains; but corn, rye and malted barley are the principle grains used. Whisky is an alcohol distillate from a fermented mash produced at less than 190° proof in such a manner that the distillate possesses the taste, aroma and characteristics generally attributed to whisky; stored in an oak container, and bottled at not less than 80° proof. Also, whisky may contain mixtures of other distillates for which no specific standards of identity are noted.

Bourbon, rye and wheat whiskies are produced (distilled) at a proof no higher than 160° from a fermented mash of not less that 51% corn, rye or wheat and aged in new, charred oak barrels at a proof no greater than 125°. Also, these whiskies may include mixtures of whiskies of the same type. Corn whisky differs in that it may be aged in used or uncharred new oak barrels. Also, corn whisky may include a mixture of other whiskies. Tennessee whisky has the same definition as the other four whisky types, but to be labeled 'Tennessee' it must be produced and aged in wood in the state of Tennessee.

All whiskies conforming to Section 5.22 of the BATF regulations must be aged a minimum of two years. To be designated a 'straight' whisky, it must conform to all regulations for its type and be aged not less than two years. 'Light' whisky is another type of whisky produced in the US. It is distilled at more than 160° but less than 190° proof and aged at least two years in used or uncharred new oak barrels.

In the US regulations, neutral spirits, vodka, Scotch whisky, Irish whiskey, and Canadian whisky are further defined. Neutral spirits are distilled spirits produced from any material distilled at or above 190° proof and bottled at not less than 80° proof. Vodka is a neutral spirit distilled and treated with charcoal or other materials to be without distinctive character, aroma, taste or color. Scotch, Irish and Canadian whiskies are defined as 'distinctive products of Scotland, Ireland, and Canada, respectively, and produced and distilled under the laws of those countries'.

History of North American whisky production

Whisky production began in the US in 1733 when the British government passed the Molasses Act. Until that time, the colonists produced distilled spirits from molasses. The Molasses Act imposed a duty on molasses of non-British origin. Since the American colonists imported most of their molasses from the French and Spanish islands, they were greatly concerned. Since non-British molasses was cheaper and more abundant, smuggling and ignoring the Molasses Act (and the later Sugar Act) was the basis for much of the 'Spirit of '76'.

Pre-revolution grain whisky production was small; although history notes that settlers in western Maryland and Pennsylvania produced rye whisky from their abundant rye grain crops and that rye whisky began to replace the popular molasses-based rums. After the Revolution, the Embargo Act cut off the supply of molasses; and with abolition of the slave trade by the new Congress, both molasses and slaves were smuggled into the US. These events increased the cost of molasses and accelerated the decline of rum.

THE WESTWARD MIGRATION

Early settlers crossing the Allegheny Mountains included many Scots and Irish immigrants who were grain farmers and distillers with knowledge of pot still operation from their homelands. They produced the rye whisky that became the first 'American' whisky. When Alexander Hamilton needed money to pay the debts incurred during the American Revolution, he pushed an excise tax levied on distilled spirits through Congress. As news of the tax spread, the uproar and public outrage was so intense that President Washington sent 13,000 troops into western Pennsylvania to quell the 'Whisky Rebellion'. As the troops entered from the east, many farmer-distillers packed their stills and headed west to Kentucky to avoid both the tax and the army.

The farmers found Kentucky soils not as suitable for rye and wheat crops as soils in Pennsylvania and Maryland. They discovered that corn was much easier to cultivate. The first writing that expounded on corn growing in Kentucky comes from the Jesuit Hierosm Lalemont. He noted 'to mention the Indian Corn only, it puts forth a stalk of such extraordinary thickness and height that one could take it for a tree, while it bears ears two feet long with grains that resemble in size our large Muscatel grapes' (Carson, 1963).

Whisky production grew rapidly in the early frontier areas as the settlers found in whisky a means of moving grain to market. A pack horse could carry only four bushels of corn, rye or wheat; but that same horse could carry 24 bushels of grain that had been mashed and distilled into two kegs of whisky. Also, the price of whisky was more than double the price the farmer could get for grain.

BOURBON'S 'ACCIDENTAL' HISTORY

As settlements moved westward, the demand for spirits increased. Riverboats had become a means for shipping barrelled whiskies to their destinations. A version of 'bourbon history' recounts how a Baptist minister, Elijah Craig, burned or 'charred' the inside of fish barrels to rid them of the fishy smell so he could fill the barrels with whisky to be shipped by raft down the Mississippi River to New Orleans. The whisky from the charred oak barrels 'aged' during shipping and storage. This aging improved the character of the whisky, gave it color and smoothed the taste (Carson, 1963).

Another version of this history tells of a careless cooper who accidentally let the staves catch fire (char) when heating them for pliability to make into barrels. Not wanting to lose money, he did not tell his distiller customer about the charred staves in the barrels. Months later, after the distiller filled the barrels and shipped the whisky downriver, the distiller heard pleasing compliments about his whisky. After discovering the cooper's 'mistake', the distiller asked him to repeat the charring process for all of his barrels.

Contrary to popular belief, none of this 'history' occurred in Bourbon County, Kentucky, and no one really knows how bourbon whisky was first made. The only historical evidence indicating Bourbon County as the source of bourbon whisky comes from a

1787 indictment of James Garrad (later a Kentucky governor) and two others by a Bourbon County grand jury for retailing liquor without a license. The only certainty in any of the lore is that Kentucky has a county named Bourbon and produces a whisky by the same name (Connelley and Coulter, 1922).

ESSENTIAL TRADITIONS

Despite uncertainty about origins of the bourbon name, a tradition of good whisky making was handed down from fathers to sons for generations. Formulas, mash bills, yeasting methods and skills for operating the stills were passed along, even though many farmer-distillers could not read or write. They did not know acrolein from fusel oil, but they did have the special knack for making the 'cuts'. They knew good, clean yellow corn and plump rye. They faithfully guarded their yeast and yeast methods though many could not have said whether yeast belonged to the animal or vegetable kingdoms. Two exceptional bourbon whiskies of the 19th century were Old Taylor and Old Crow. Old Overholt was reportedly the best of the rye whiskies.

During the 19th century, the pot still evolved into the continuous column 'beer still' with a doubler or thumper. The continuous still operations allowed distillers to move to larger fermentors, more and larger cookers and automated grain handling. As the distillery operations grew and became increasingly automated, some of the smaller distilleries fell by the wayside or promoted their brands as better as a result of their 'old time, small distillery tradition and quality'. They touted this tradition and sold it as part of the product. The well known Maker's Mark bourbon is a prime example of tradition and sound practice bottled and successfully marketed to modern consumers.

As grain whisky and bourbon production grew in the 19th century, the US government increased the excise tax and the number of regulations. Costs passed on to the consumers dampened their enthusiasm for drink; but government regulation incensed leaders of the Temperance Movement because the tax and regulations drew attention to the production and sales of liquor. Most whisky at that time was sold 'from the barrel', and quality standards were almost nonexistent. It was not until the end of the century that consumers could purchase whisky sold in a corked and sealed bottle. Old Forester was the first product with 'guaranteed quality' put on the label. Old Heritage was the first bourbon with a strip stamp over the cork, thereby becoming the first 'bottled-in-bond' bourbon.

THE PROHIBITION ERA

All of the improvements in the 'character' of whisky production and consumption were to no avail when at 12:01 a.m. on Saturday, January 17, 1920 all beverages containing more than 0.5% alcohol were outlawed in the US (Tennessee, the state with the first registered distillery in the country, Jack Daniel Distillery, became the third state to vote to go dry in 1910, ten long years before the passing of the Volstead (Alcohol Prohibition) Act). The 18th amendment to the Constitution, which prohibited the production and sale of alcoholic beverages, sounded a death knell for many distilleries. A drive through the countryside revealed closed distilleries choked with weeds with facilities in ruins. Brown-Forman Distillery in Louisville, Kentucky, was one of the few that survived because it produced its Old Forester Bourbon 'for medicinal purposes only'. Other distilleries lay in wait for 'the experiment' of prohibition to end.

Passage of the 21st amendment ended Prohibition after 13 years. At midnight on April 7, 1933 wines and beer were again legally sold; and on December 5 of that year at 5:32 p.m. bourbon was again on the market. The American distilled spirits industry surged into production, building larger, more modern distilleries. The resumed legal production and sales were reinforced by the federal government when it passed the Federal Alcohol Control Act, which eliminated the sale of bulk whiskies to the wholesale and retail trades. This Act also formed the Alcohol and Tobacco Tax Division of the Internal Revenue Service. Though creating bureaucracy and taxation, the new Act also set regulations and definitions for producing spirits in the United States.

DISTILLATION'S NEW ERA

The new regulations defined the production of bourbon, rye, corn and blended whiskies as well as gin, brandies, rums, cordials and vodka according to their spirit type. The Act also noted the use of geographical designations with an origin defined for Scotch, Canadian, and Irish whiskies. This evolution of the industry led to the organization of companies that had goals of producing top quality spirits within the new regulations. New companies such as National Distillers and Schenley joined the American distilleries that survived prohibition, Brown-Forman and Jim Beam, along with the Canadian distillers, Seagrams and Hiram Walker. These distillers based their production methods on precisely defined procedures from yeasting to maturation.

During World War II, North American distilleries ceased whisky production and began manufacturing industrial alcohol for the war effort. Distillers gained the resources for further technical improvements; and at the War's end, the industry in the United States was technically ready to produce better bourbons, ryes, and Tennessee whiskies than ever before.

While the basis for modern American whisky production was developed during World War II, today's modern American distillery operates with recent innovations. Even with all of the modern technology, the US distiller still carefully controls his yeasts, mash bills, distillation methods and maturation criteria, essential factors for good quality products initiated by the early pioneer distillers.

Production and maturation operations

In the production of American whiskies, six factors determine the character and flavor for each type of whisky:

1) Grain proportions in the mash bill
2) Mashing technique
3) Strain of yeast
4) Fermentation environment
5) Type and operation parameters of distillation equipment
6) Type of barrel used and the maturation process

These factors were recognized and carefully controlled in the distilling operations of the early settlers (Lyons, 1981). Families and companies that ensured consistency and control over these factors remain in business today. Those who failed to strictly adhere to a regimen controlling each factor have fallen by the wayside.

A MATTER OF DISTINCTION

Popular bourbon producers use specific process differences to make their products distinct. The Jack Daniel Distillery continues to produce its whisky with the same processes used more than 100 years ago. Their strict adherence to tradition along with a down-home, folksy image have proven successful marketing tools for the whisky. At Maker's Mark, grain selection and proportions are used to produce a superior whisky. They use wheat instead of rye in the mash bill. The wheat and good, consistent control of all factors, especially the barrels, make Maker's Mark the smoothest of the bourbons. Their claim of 'handmade' is justified because of their intense attention to production parameters.

All American whiskies maintain standards for the six production factors; and the variations among distilleries in adherence to standards for these factors determine flavor and cost differences. All American distillers start with a careful grain purchasing program. Though price is a criterion, they all use No. 1 or No. 2 yellow corn, No. 1 plump northern rye, and choice northern malted barley. Any off odors or below-grade grain are rejected at the distillery. Very stringent grain standards are a common feature of all American distilleries (Table 1).

MASH BILL

The mash bill may vary, with a typical bourbon having a mash bill of 70% corn, 15% rye and 15% malt. A typical Tennessee whisky may have 80% corn, 10% rye and 10% malt while a typical rye whisky will have a mash bill of 51% rye, 39% corn and 10% malt. All grains are

Table 1. Specifications and analyses of corn, rye, wheat and malt.

	Specification		*Typical analysis*
Corn (No. 2 recleaned)			
Grain odor	No musty, sour or off odor		'Meets spec'
Moisture, %	14.0	(maximum)	12-14
Cracked grains and foreign material, %	2.0	(maximum)	1-2
Damaged kernels, %	3.0	(maximum)	0-1.5
Heat-damaged kernels, %	0.2	(maximum)	0-0.1
Bushel weight, lbs	55.0	(minimum)	55-60
Rye (No. 1 plump)			
Odor	None	'Meets spec'	
Moisture, %	14.0	(maximum)	10-14
Thins, %	2.0	(maximum)	1-2
Dockage, %	2.0	(maximum)	1-2
Bushel weight, lbs	56.0	(minimum)	56-60
Malt			
Bushel weight, lbs	35.0	(minimum)	35-38
Moisture, %	6.0	(maximum)	4-6
α-amylase	60.0	(minimum)	60-64
Diastatic power	22.0	(minimum)	22-26
Bacteria count, CFU/g	1,000,000.0	(maximum)	400-500,000
Wheat			
Odor	None	'Meets spec'	
Moisture, %	14.0	(maximum)	10-14
Thins, %	2.0	(maximum)	1-2
Dockage, %	2.0	(maximum)	1-2
Bushel weight, lbs	56.0	(minimum)	56-60

ground, with the hammer mill being the most common type of processing; however some roller and attrition mills are still in use. The milling (Figure 1) is checked for grind by a sieve analysis. A typical sieve analysis for grains in a bourbon mash bill is shown in Table 2.

Table 2. Typical sieve analysis for grains in a bourbon mash bill.

US Sieve #	*Corn*	*Rye*	*Malt*	*Wheat*
16	15	22	2	20
20	21	25	8	26
30	17	13	14	12
40	13	9	16	8
50	10	7	13	6
60	3	2	8	2
Through 60	21	22	34	22

Mashing

Mashing techniques vary considerably, but the major difference is whether pressure or atmospheric batch cooking is used. Bourbon, rye, wheat, Tennessee and corn whisky are mashed using batch cookers. Only the 'blend' or 'light' whisky producers use continuous cookers. Pressure cooking is usually done at 124°C while atmospheric cooks are done at 100ºC. Cooking time varies from 15 minutes to 1 hr. Conversion time and temperature are very consistent among distilleries. Malt is never subjected to temperatures greater than 64ºC; and conversion time is usually less than 25 minutes to minimize contamination. All distillers use backset (centrifuged or screened stillage from the base of the still), but the quantity of backset will vary based upon the beer gallonage (gallons of water per 56 lb distillers bushel of grain) to be used. American whiskies have beer gallonages in the 30-40 gallon range. High energy costs for by-product recovery have encouraged some distillers to use lower beer gallonage ratios for spirits. However, bourbon and Tennessee whisky producers continue to use 30-40 gallon beers. The cooling of cooked mash

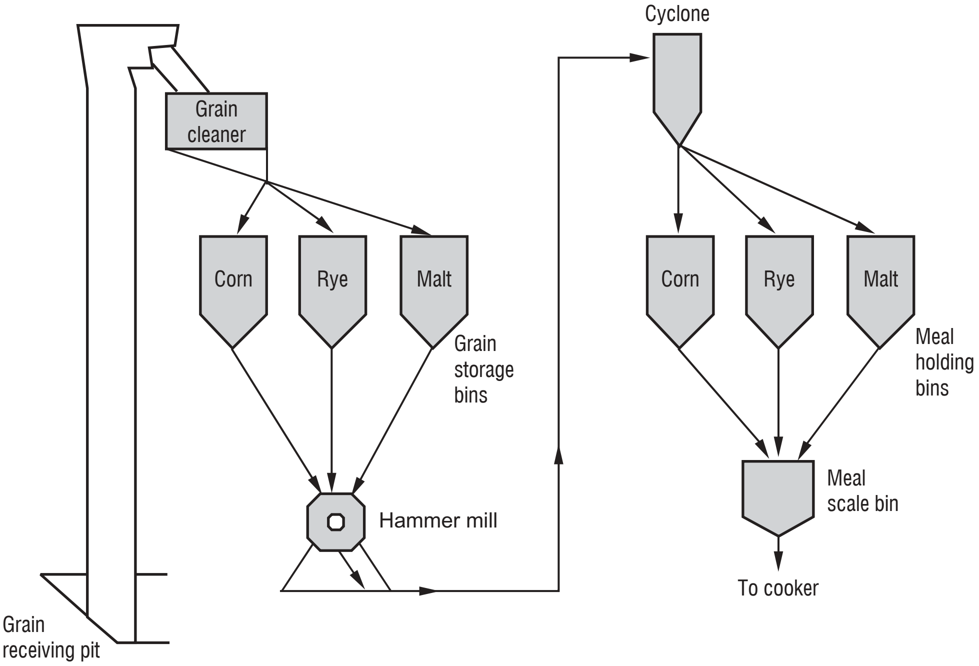


Figure 1. Typical grain-handling and milling facility.

to fermentation temperature is achieved using vacuum (barometric condensers) or cooling coils.

YEASTING

All whisky producers use *Saccharomyces cerevisiae*, however the yeasting techniques vary tremendously between the 'modern' and 'traditional' distillers. The modern distillers have elaborate yeast laboratories and will propagate a new yeast from an agar slant every week. They are very aseptic and accurate, assuring continuity of the same flavor. The 'traditional' distillers use yeast stored in jugs; and though they backstock weekly, the potential for gradual yeast culture changes and contamination can lead to flavor variances. These distillers take extra effort and care to ensure that their yeasting does not cause ester, aldehyde or fusel oil variances in the distillate.

The most common grains used for yeasting are small grains, rye and malted barley. These grains are cooked in a separate cooker to about 63ºC, and the pH is adjusted to 3.8 with lactic acid bacteria grown in the yeast mash. Lactic acid production is then stopped by increasing the temperature to 100ºC for 30 minutes to kill the bacteria. This aseptic, sterile mash is then ready for the yeast from the dona tub grown in the laboratory (Figure 2). The yeast fermentation temperature is controlled at 27-30ºC; and the yeast propagates until the Balling drops to half the original 22º Balling reading. This yeast mash will have a yeast concentration of 400 million cells/ml. Both modern and traditional distillers regularly have clean, sterile yeasts free of bacterial contamination that may cause side fermentations and unusual congeners in the distillate. The 'lactic souring' and the alcohol content of the finished yeast mash (8%), along with sterile dona and yeast tank methods contribute to the excellent reputation American whiskies have for fermentation congener consistency. The advantage of using small grains are: preservation of enzymes for secondary conversion, low steam requirements and shorter processing time. Also, because of its nutrient value, barley malt is the most important constituent of yeast mashes. Corn is not used in a yeast mash because it does not contain the growth factors required for yeast and lactic bacteria growth.

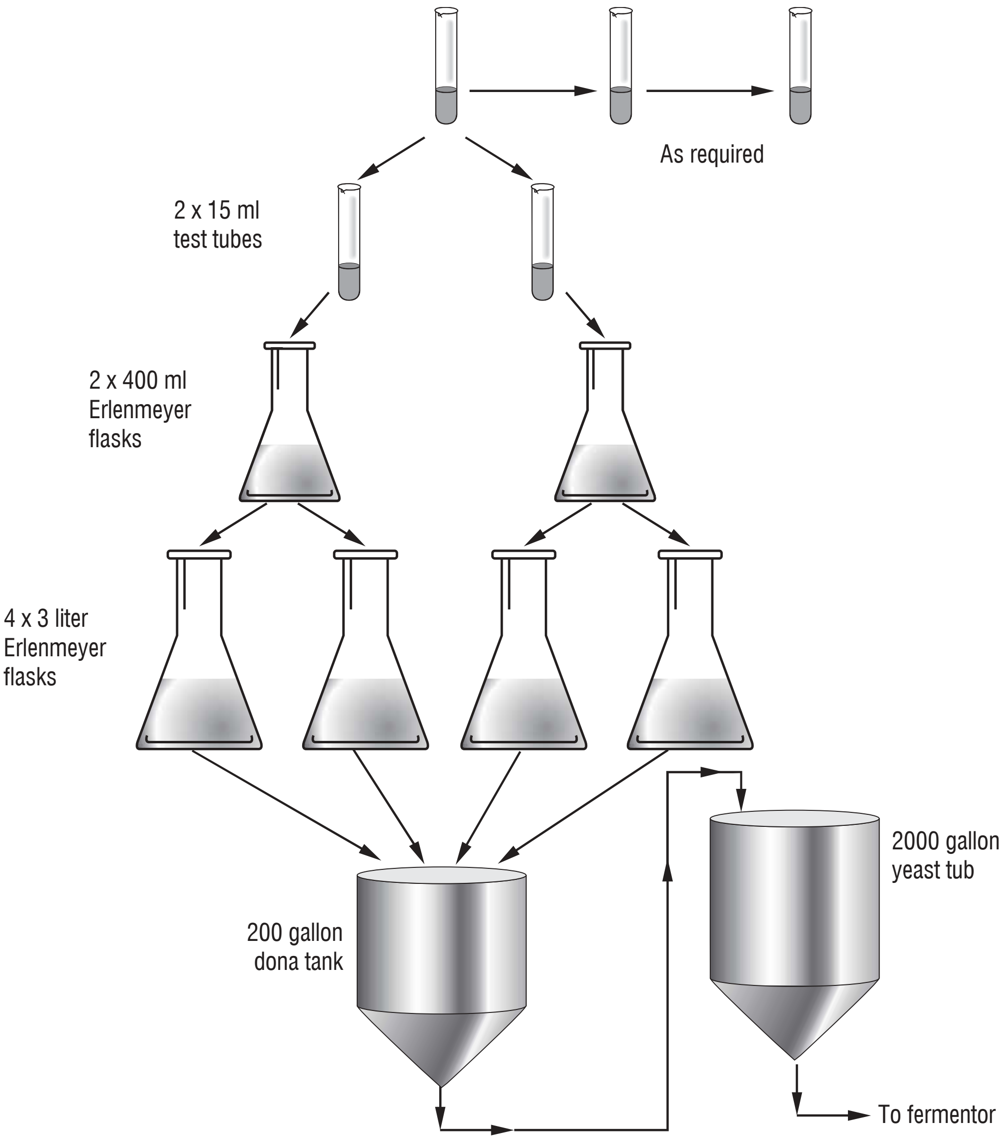

As required
2 x 15 ml
test tubes
2 x 400 ml
Erlenmeyer
flasks
4 x 3 liter
Erlenmeyer
flasks
2000 gallon
yeast tub
200 gallon
dona tank
To fermentor

Figure 2. Stages in yeast production.

FERMENTATION

Fermentation is the simplest part of the production process, but requires more control with efficient equipment in order to have stable, consistent results. After the two or three cooks required are completed, cooled, and transferred to the fermentor, the fermentor is 'set'. Setting the fermentor means filling the fermentor with cooked mash, inoculated yeast and backset. The yeast mash is pumped in as soon as the first cook is added to the fermentor. The addition of backset and/or water is done at the end of filling to bring the fermentor to the desired final beer gallonage. Most distillers use a 30-36 gallon mash, and the water:backset ratio determines the set pH. Set pH values of 4.8-5.2 are considered to be the best starting point. The modern distillers have closed-top fermentors with cooling coils or external heat exchangers to control fermentation temperature. They usually set fermentors at 27-29ºC and control at 30-31ºC. Traditional distillers will have metal or wood open-top fermentors without any device to control fermentation temperature. They usually set their fermentors as cool as they can (18-21ºC) and let the fermentors work up to 31-32ºC. All of this is controlled by mash cooker cooling, addition of cold water and weather factors. Contamination is controlled by cleaning fermentors, ensuring no mash pockets in pipes and regular steaming of fermentors.

Both traditional and modern distillers ferment their beers for at least 72 hrs, and some for as long as 96-120 hrs. Three and five day fermentations are the norm. During these periods the Balling will drop to 0.0 and the pH from 5.0 to 3.8 while the alcohol concentration rises to 8-10%. All the changes that happen during fermentation are checked daily by performing 'beer chemistry'. Balling, pH, acids, and fermentor temperature are monitored and recorded daily. The pH is the main indicator of contamination and potential fermentation problems and is regularly measured by traditional and modern distillers (Table 3).

DISTILLATION

Upon completion of the fermentation process, the beer with 8.0-10.0% alcohol is transferred from the fermentor to the beer well. The beer well is a holding tank for the fermented beer, such that a continuous feed to the beer still can be maintained. Beer wells are usually 1-1.5 times the size of a fermentor. They also have continuous agitation to prevent solid grain particles from settling to the bottom of the vessel. All American whisky producers use a continuous still (Figure 3), though some have a second distillation 'doubler' or 'thumper' (Figure 4). The basic difference between a doubler and a thumper is whether the unit is operated with a liquid level (doubler) or essentially dry (thumper). Both the doubler and the thumper provide a second distillation.

The beer is pumped into the upper section of the first continuous column, the beer still, six to ten plates from the top. Live steam is introduced at the bottom. Beer stripping plates 1-18 have perforations, a downcomer from above and a dish on the plate below to hold the beer liquid at a set level so the plates are never dry, as the beer moves back and forth across each plate. The steam passing up and through the perforations, controlled by pressure or flow rate, strips the lighter, more volatile alcohol from the

Table 3. Typical analysis of beers from bourbon, rye and corn whisky production.

	Bourbon	*Rye*	*Corn*
Set sample			
Balling	13.4	13.4	12.3
Titratable acidity	4.5	3.6	2.8
pH	4.5	5.0	5.2
Temperature, °C	27.0	24.0	27.0
24 hr sample			
Balling	2.6	4.0	3.6
Titratable acidity	5.1	4.6	4.2
pH	4.2	4.4	4.3
Temperature, °C	30.0	31.0	29.0
48 hr sample			
Balling	2.4	3.6	1.0
Titratable acidity	7.8	7.5	6.1
pH	3.8	3.9	3.8
Temperature, (°C)	30.0	30.0	30.0
Drop sample			
Balling	0.4	1.5	-0.4
Titratable acidity	8.2	7.9	7.1
pH	3.8	3.9	3.8
Temperature, °C	30.0	30.0	30.0
Alcohol, % by volume	6.73	5.8	6.8
Residual carbohydrates, %	8.0	8.4	4.2
Residual carbohydrates, % maltose	0.73	0.6	0.46

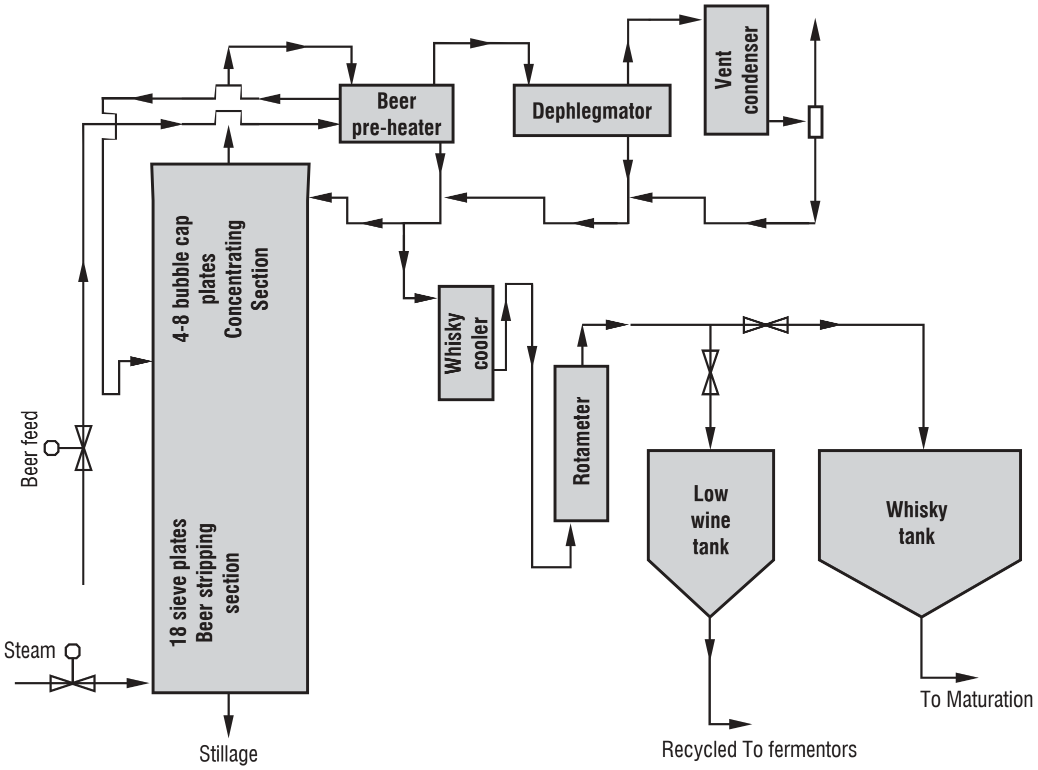

Beer pre-heater
Dephlegmator
Vent condenser
4-8 bubble cap plates Concentrating Section
18 sieve plates Beer stripping section
Beer feed
Steam
Stillage
Whisky cooler
Rotameter
Low wine tank
Whisky tank
To Maturation
Recycled To fermentors

Figure 3. Bourbon whisky still.

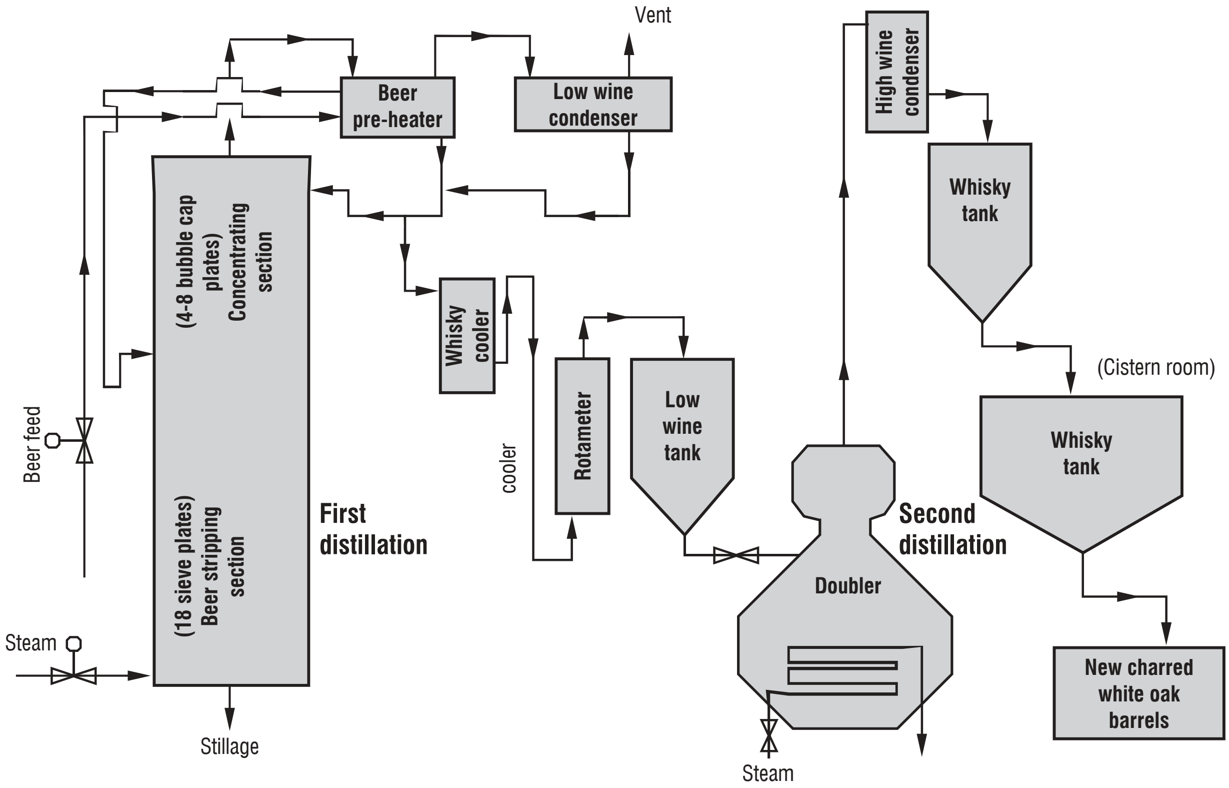

Vent
Beer pre-heater
Low wine condenser
High wine condenser
Whisky tank
(4-8 bubble cap plates) Concentrating section
(18 sieve plates) Beer stripping section
Beer feed
Steam
Stillage
First distillation
Whisky cooler
cooler
Rotameter
Low wine tank
(Cistern room)
Whisky tank
Second distillation
Doubler
Steam
New charred white oak barrels

Figure 4. Bourbon whisky distillation system, including doubler.

water/grain mixture on the plates. When this alcohol gets to the top 4-8 bubble cap plates it is concentrated to about 100° proof (50° GL). As the vapors continue up into the beer preheater (a heat exchanger to heat beer going into the column), some alcohol is condensed and refluxed to the top of the beer column. The rest flows to the doubler or thumper as vapor. From either of these pot still-type chambers, the vapor goes to the condenser where the product is drawn off at 130-140° proof (65-70° GL). Bourbon cannot be distilled above 160° proof (80° GL). If it comes off at more than 160° proof, it must be called 'light' whisky. The stillage from the base of the beer still is pumped to the dryer house to be processed. The 'high wine' from the stills is pumped to the cistern room where it is held, tested and reduced to barrelling proof (Table 4).

BY-PRODUCT RECOVERY

The by-product recovery does not usually receive the same care that the other processes demand. The only essential for this process is ensuring that the backset stays hot, around 99ºC, so that it is absolutely sterile when used in the mash tubs, yeast tubs and fermentors. (Most distillers use 20-30% backset in their total process.) Furthermore, the hotter the backset remains, the greater the savings in energy costs during the cooking process. The stillage from the base of the beer still has about 7-10% total solids. When used for backset it is screened or centrifuged to prevent solids accumulation in the cooker and fermentors. Nearly all distillers have a dryer house, though a couple of traditional distillers continue to sell their 'slop' to nearby farmers. For a while some, like the Jack Daniel Distillery, fed the stillage to cattle in wet-feeding operations; but environmental restrictions have generally eliminated such practices. Also, modern dryer house operations have become very profitable and the profit helps lower the cost of making whisky.

COOPERAGE AND MATURATION

Cooperage and maturation are the processing factors that distinguish bourbon and American whisky from the other whiskies of the world. Only bourbon whisky regulations require that it be matured in a new charred, white oak barrel. Other whiskies of the world may require the use of small wooden barrels, but no other whisky but bourbon goes through the care and expense of requiring small white oak barrels to be freshly charred.

The making of the bourbon whisky barrel is a very traditional, but exact science and craft. Staves and heading are quarter-sawed from mature, white oak timber. Actually, some physical variance exists within a single tree, but no more than between trees. After being quarter-sawed with the medullary rays not less than 45 degrees to the stave surface, the staves and heading are air-dried in a stave yard. The more traditional distilleries require wood to be air-

Table 4. Typical operating data for whisky distillations.

	Bourbon	*Rye*
Product proof	130°	130°
Still pressure (inches of water)	48	42
Steam rate, lb/hr	12,000	12,000
Beer feed rate, gallons/minute	120	117
Reflux rate from beer preheater, gallons/hr	350	300
Reflux rate from dephlegmator, gallons/hr	700	750
Reflux rate from vent condenser, gallons/hr	30	65
Draw-off of product, gallons/minute	12.5	10.0
Still losses, %	0.0004	0.00025
Water temperature to vent condenser, °C	21	21
Water temperature from dephlegmator, °C	79	79
Beer solids, %	0.06	0.06

dried at least one year. The modern distillery usually has no air-drying specifications and allows its barrels to be produced from wood that has been air-dried for six months or less. All of the wood is kiln-dried at the cooperage, with staves and heading dried to 12 and 10% moisture, respectively. The kiln-drying is essential to prepare the wood for the planing, milling, edging and joining operations that cannot be done on wet wood. More important, however, is that proper drying (air-drying a year followed by kiln-drying), makes the wood chemistry satisfactory for flavoring the whisky during maturation.

As the whisky goes through maturation (3-4 years for the modern distillers and 4-8 years for the traditional distillers), two distinct types of reactions occur: reactions between the distillate components (regardless of the barrel); and reactions that occur when the distillate extracts chemical compounds from the wood. A major factor differing among the distillers is the warehouse environment. Most modern distillers heat their warehouses in winter. One particular distiller even controls the heat cycles to ensure constant aging. The traditional distillery seldom has heated warehouses and depends strictly on Mother Nature to determine the number and range of its heating and cooling cycles.

Whether the heat cycle is natural or forced, the greatest rate of change or formation of congeners occurs in the first 12-16 months. Only ester formation occurs at a fairly constant rate. Proof increases at a fairly constant rate of 4-5% each year of aging. Other specific changes occuring when the distillate reacts with the charred wood are: a) aldehyde formation, specifically acetaldehyde, which comes from the alcohol via oxidation, b) acetic acid formation with greatest activity in the first year of maturation, and c) ester formation (ethyl acetate) from the alcohol via oxidation. The components coming from the wood are tannins, sugars, glycerol and fructose. The hemicellulose in the wood appears to be the source of the sugars found in aged American whiskies.

The depth of charring and 'toast level' in the barrel determines the color of the whisky. Color formation is almost instantaneous when the distillate is put into the charred barrel, with 25-30% of the color formed in the first six months. Some color development occurs each year of maturation until the whisky is dumped from the barrel. The final product is then filtered, its proof reduced with demineralized water and bottled. Compared to the sweet rums, 'breathless vodka', fruity gins, light Canadians and Scotches, the American whiskies have flavor and bravado with a balance that is pleasant to the taste. American whisky produced and matured as described has a big, pungent aroma that leaves no doubt that it is bourbon or Tennessee whisky.

Though the modern and traditional distillers have different levels of technology, they both use the same basic processes for making bourbon or other American whiskies. Individual plant nuances produce different flavors, but they all have an American whisky bouquet and taste.

References

Carson, G. 1963. The social history of bourbon. Dodd, Mead & Company, New York.

Connelly, W.E., and E.M. Coulter. 1922. History of Kentucky, Vol. II. The American History Society, Chicago and New York.

Lyons, T.P. 1981. Gasohol, A Step to Energy Independence. Alltech Technical Publications, Nicholasville, Kentucky.

Contamination and hygiene

Chapter 20

Bacterial contamination and control in ethanol production

N.V. NARENDRANATH

North American Biosciences Center, Alltech Inc., Nicholasville, Kentucky, USA

Introduction

Bacterial contamination is a major cause of reduction in ethanol yield during fermentation of starch-based or sugar-based feedstocks by *Saccharomyces cerevisiae*. The sugar consumed by bacteria is diverted away from alcohol production and is converted into by-products. In addition to reducing the yield, the presence of bacterial metabolites in the fermentation medium is inhibitory to yeast growth and metabolism. In distilleries, cleaning and sanitizing are much less rigorous compared to breweries, and mashes are subjected to less heat and are not sterile. Contaminants can arise from tankage, transfer lines, heat exchangers, raw materials, active dry yeast, poorly stored backset, or yeast slurry used as inoculum. Microbial numbers can be significantly reduced by cleaning and sanitizing the equipment, by maintaining backset at a temperature over 70°C, by pasteurizing or chemically sterilizing the substrates, and by adding antibiotics to fermentors (Ralph, 1981). In a distillery, it is necessary to recognize the potential sources of contamination and know the most commonly encountered contaminants so that appropriate measures can be taken to minimize serious losses.

Commonly encountered contaminants

The bacterial contaminants encountered during alcohol production include both Gram-positive and Gram-negative species. Figure 1 summarizes the types of bacteria that can occur in a distillery and their characteristics. Among the bacterial contaminants encountered, lactic acid bacteria are the most troublesome because of their tolerance to high temperature and low pH and their ability to grow rapidly and survive under ethanol production conditions. Therefore, discussion in this chapter will primarily focus on the effects of lactobacilli, the predominant organism in distilleries and fuel ethanol plants and their control.

ACETIC ACID-PRODUCING BACTERIA

While Gram-positive lactobacilli comprise the most important single group of bacterial contaminants, certain Gram-negative bacteria such as acetic acid bacteria cannot be ignored. The genera *Acetobacter* and *Gluconobacter* comprise bacteria that produce acetic acid as the dominant end product of metabolism. *Acetobacter* oxidizes acetic acid to CO_2 and water whereas *Gluconobacter* just produces

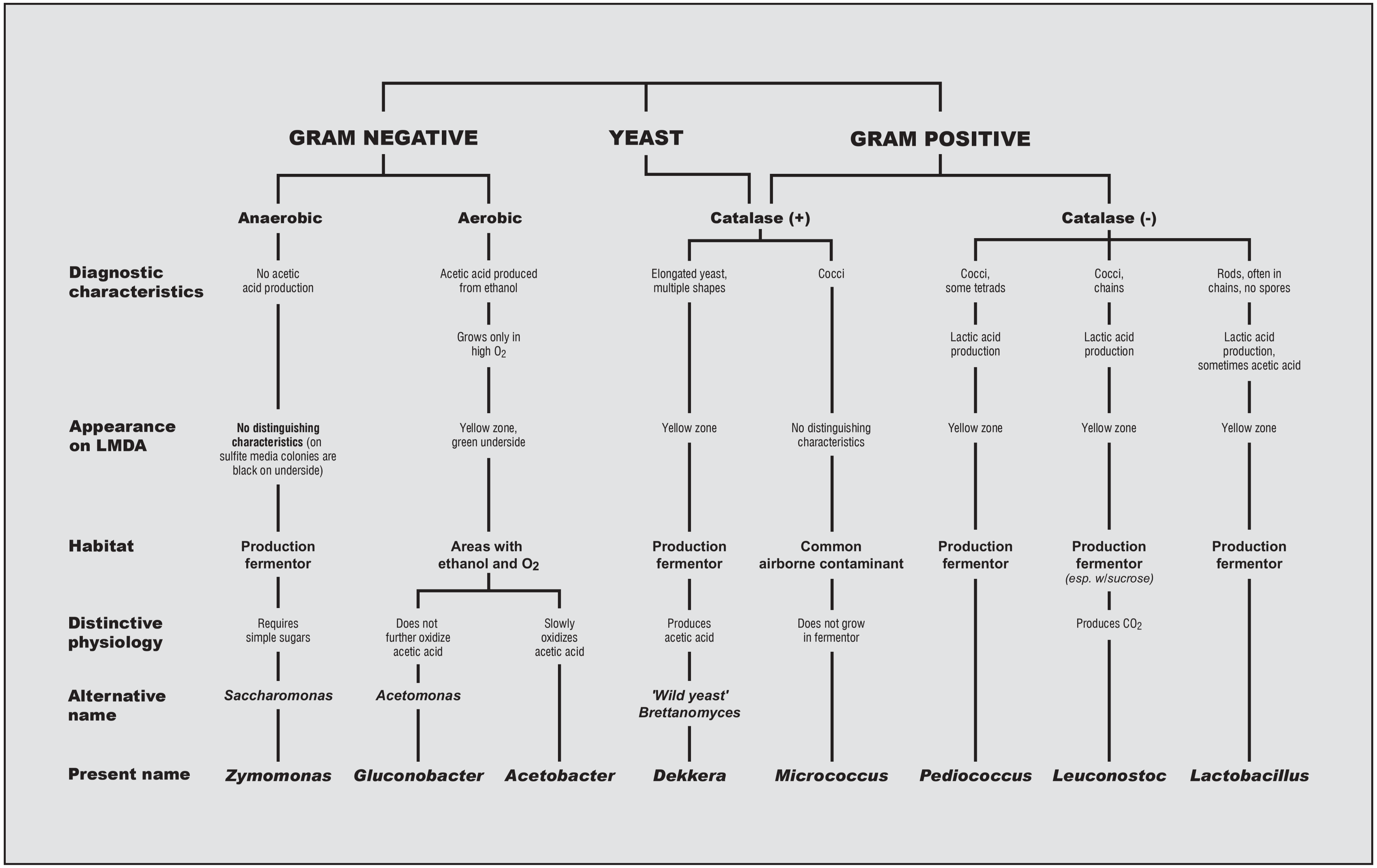

GRAM NEGATIVE
YEAST
GRAM POSITIVE
Anaerobic
Aerobic
Catalase (+)
Catalase (-)
Diagnostic characteristics
No acetic acid production
Acetic acid produced from ethanol
Grows only in high O_2
Elongated yeast, multiple shapes
Cocci
Cocci, some tetrads
Cocci, chains
Rods, often in chains, no spores
Lactic acid production
Lactic acid production
Lactic acid production, sometimes acetic acid
Appearance on LMDA
No distinguishing characteristics (on sulfite media colonies are black on underside)
Yellow zone, green underside
Yellow zone
No distinguishing characteristics
Yellow zone
Yellow zone
Yellow zone
Habitat
Production fermentor
Areas with ethanol and O_2
Production fermentor
Common airborne contaminant
Production fermentor
Production fermentor (esp. w/sucrose)
Production fermentor
Distinctive physiology
Requires simple sugars
Does not further oxidize acetic acid
Slowly oxidizes acetic acid
Produces acetic acid
Does not grow in fermentor
Produces CO_2
Alternative name
Saccharomonas
Acetomonas
'Wild yeast' Brettanomyces
Present name
Zymomonas
Gluconobacter
Acetobacter
Dekkera
Micrococcus
Pediococcus
Leuconostoc
Lactobacillus

Figure 1. Classification and characteristics of common contaminants encountered in a distillery.

acetic acid from sugar substrates and does not further oxidize the acetic acid produced.

Acetic acid bacteria are rod-shaped cells and are unable to survive and grow in the absence of oxygen. Therefore, these organisms are not a threat in the fermentor where the conditions are anaerobic. However, acetic acid bacteria can occur in the yeast propagator or yeast conditioning tank, which is subjected to high agitation and aeration to promote yeast growth. Once the inoculum is pumped to the fermentor and an anaerobic system is established, acetic acid bacteria will usually die; however sufficient acetic acid to slow or inhibit yeast growth may already be present. It is easier to control these bacteria in batch propagation systems where the tank is emptied, cleaned and sanitized between batches.

LACTIC ACID BACTERIA

Lactic acid bacteria (LAB) are Gram-positive, catalase negative, microaerophilic or aerotolerant anaerobes. They are either rod-shaped or cocci-shaped cells that produce lactic acid as a major end product of carbohydrate metabolism. LAB have complex nutritional requirements (Kandler and Weiss, 1986). These bacteria can grow in a wide range of temperatures, from 2 to 53°C, with an optimum temperature generally between 30 and 40°C. Optimal pH for growth is 5.5-6.0. Growth generally occurs at pH 5.0 or less, but growth rate is significantly reduced. Under optimal growth conditions, the doubling time for lactic bacteria is much quicker than that of yeast.

Metabolically, LAB possess efficient carbohydrate fermentation pathways. The main fermentation pathways for glucose are the Embden-Meyerhof pathway, converting 1 mole of glucose to 2 moles of lactic acid (homolactic fermentation) and the 6-phosphogluconate pathway, resulting in 1 mole of CO_2, 1 mole ethanol (or acetic acid) and 1 mole lactic acid (heterolactic fermentation). The **homofermentative** LAB produce virtually a single fermentation product, lactic acid, whereas the other LAB, called **heterofermentative**, produce other products, mainly ethanol (or acetic acid) and CO_2 as well as lactic acid. The abbreviated pathways for the fermentation of glucose by homo- and heterofermentative organisms are shown in Figure 2. The differences observed in the fermentation products are determined by the presence or absence of the enzyme aldolase, one of the key enzymes in glycolysis.

Lacking the aldolase enzyme, heterofermenters instead oxidize glucose-6-phosphate to 6-phosphogluconate and then decarboxylate

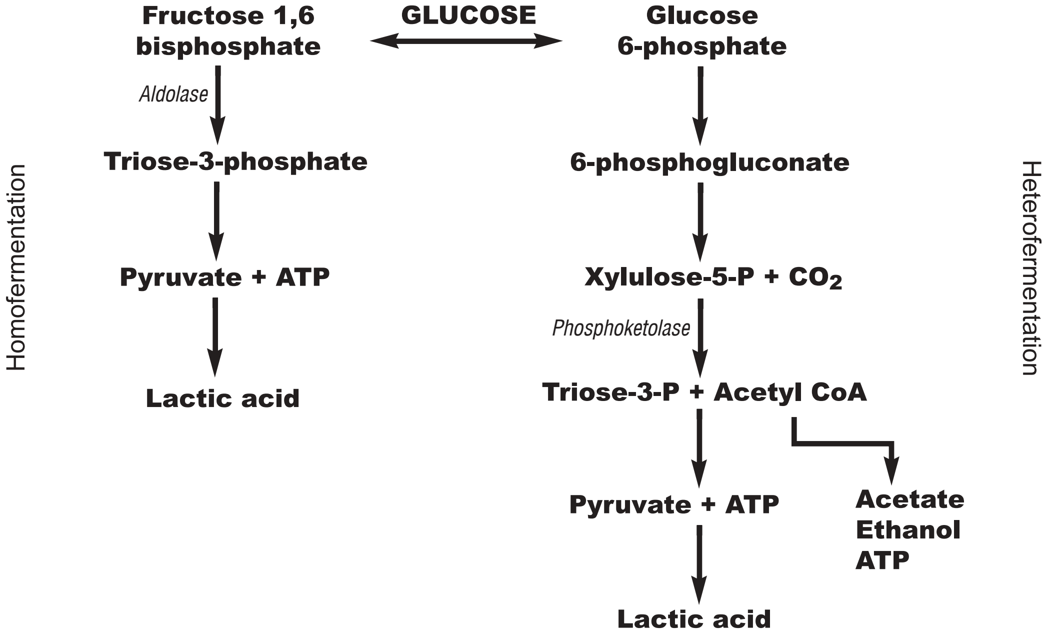


Figure 2. The fermentation of glucose in homofermentative and heterofermentative lactic acid bacteria.

this to pentose phosphate, which is then broken down to triose phosphate and acetylphosphate by means of the enzyme phosphoketolase. Whereas the hetero-fermentative lactobacilli possess ketolase but no aldolase, homo-fermentative species possess aldolase but no phosphoketolase. Obligate homofermenters are thus unable to ferment pentoses, which are broken down by the heterofermenters via phosphoketolase, yielding equimolar amounts of lactic acid and acetic acid. However, one group of homofermentative lactobacilli possesses an inducible phospho-ketolase with pentoses acting as inducers. They are thus able to ferment pentoses upon adaptation to lactic acid and acetic acid, while hexoses are homofermentatively metabolized. Therefore, these lactic bacteria are called **facultative heterofermenters**. Table 1 shows some examples of the different classes of lactic acid bacteria. Isolates of LAB from distilleries are well-adapted to the conditions existing in such fermentations (Bryan-Jones, 1975). In fact, enumeration of bacteria in many distilleries is often limited to the detection of lactic acid bacteria because aerobes and facultative anaerobes with little pH tolerance are not considered serious threats to product quality or production efficiency.

Lactobacilli effects on ethanol production by yeast

Yeasts and LAB are often encountered together in natural ecosystems and may be in competition for the same nutrients (Alexander, 1971). The genus *Lactobacillus* is of major concern to distilleries and fuel ethanol plants. Chin and Ingledew (1994) reported that *Lactobacillus fermentum* inoculated at approximately 10^8 CFU/ml did not seriously affect ethanol productivity in the fermentation of diluted (14°Plato) wheat mash. Only moderate growth (2 to 8-fold increases) of the bacteria occurred. Other scientists, however, have reported that when bacterial numbers exceeded 10^8 CFU/ml at 30 hrs of fermentation, alcohol loss was approximately 5% (Barbour and Priest, 1988; Dolan, 1979). According to Makanjuola *et al.* (1992), reduced ethanol yields, lower yeast numbers, reduced carbohydrate utilization, and an increase in acidity were all caused by the build up of lactic acid produced by lactobacilli. They found that a bacterial count of 4.5×10^8 CFU/ml at 30 hrs resulted in a 17% reduction in ethanol yield due to a stuck fermentation.

Initial bacterial contamination of mash with approximately 10^7 CFU/ml led to as much as 0.6-1% v/v reduction in ethanol depending on the strain of bacteria (Narendranath *et al.,* 1997). These authors were the first to report that both final lactic acid concentrations and decreases in ethanol yields at the end of fermentation were directly correlated with initial numbers of viable bacteria in mash (Figure 3). Apart from the diversion of glucose for growth, the production of end products such as lactic and acetic acid, and a suspected competition with yeast cells for essential growth factors in the fermenting medium are the major reasons for the reductions in yeast growth and final ethanol yield when lactic bacteria are present.

EFFECTS OF BACTERIAL END PRODUCTS ON YEAST GROWTH

The end products of metabolism of lactobacilli, lactic acid and acetic acid, are inhibitory to yeast growth and metabolism. The concentrations of acetic acid (a minor end product of heterofermentative lactic acid bacteria and wild yeasts or a major end product of aerobic bacteria such as *Acetobacter* spp.) and lactic acid inhibitory to the growth of *S. cerevisiae* were 0.5-9 g/L and 10-40 g/L, respectively, and an

Table 1. Three classes of lactic acid bacteria.

Type	Examples
Obligately homofermentative	*Lactobacillus delbrueckii*, *L. acidophilus*, *Pediococcus damnosus*
Obligately heterofermentative	*L. brevis*, *L. buchneri*, *L. fermentum*
Facultatively heterofermentative	*L. plantarum*, *L. casei*, *L. pentosus*, *L. sake*

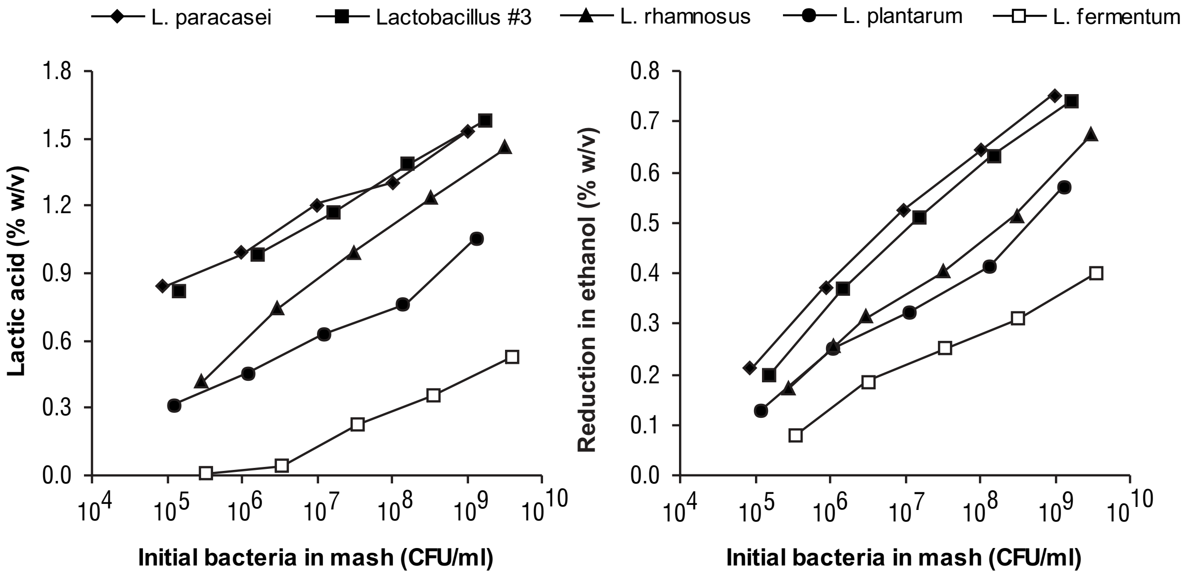


Figure 3. Effect of the initial numbers of lactobacilli on the final lactic acid concentration and on the reduction in final ethanol concentration compared to the control with no bacterial inoculation. (Adapted from Narendranath *et al.,* 1997).

80% reduction in yeast cell mass occurred at concentrations of 7.5 g and 38 g/L, respectively (Maiorella *et al.,* 1983). The effects of these acids on the specific growth rate of yeast are detailed in the chapter on continuous fermentation in this volume. The minimum inhibitory concentration (MIC) of acetic acid for yeast growth is 0.6% w/v (100 mM) and that of lactic acid is 2.5% w/v (278 mM). However, acetic acid at concentrations as low as 0.05-0.1% w/v and lactic acid at concentrations of 0.2-0.8% w/v begin to stress yeast as seen by decreased growth rates and decreased rates of glucose consumption (Narendranath *et al.,* 2001a).

The inhibitory effect of weak organic acids such as acetic acid and lactic acid on yeast growth depends on the pH of the medium, the dissociation constant of the acid, and its molar concentration. In solution, a weak acid exists in a pH-dependent equilibrium between dissociated and undissociated states, as described by the Henderson-Hasselbach equation ($pH = pK_a + \log[A^-]/[HA]$ where A^- and HA are the dissociated and undissociated species, respectively). This equation indicates that at a pH above its pK_a value, more than 50% of the acid is dissociated and that the concentration of undissociated acid increases logarithmically as the pH declines. Since organic acids are generally more toxic to microorganisms at low pH, it is assumed that the antimicrobial activity of these acids is the result of an increased proportion of undissociated molecules (Salmond *et al.,* 1984). Therefore, acetic acid ($pK_a = 4.74$) with a higher pK_a value is more toxic to yeast than lactic acid ($pK_a = 3.86$) at any given pH of the medium. Acetic acid has between two and four times more molecules in the undissociated form over a pH range between 4.0 and 4.6 compared to lactic acid (Lindgren and Dobrogosz, 1990). Undissociated weak acids diffuse passively into the microbial cell until equilibrium is established across the membrane. As molecules enter the cytoplasm, they dissociate at the higher intracellular pH. The protons liberated are either pumped out of the cell in exchange for cations or are neutralized by the buffering capacity of the cytoplasm (Booth and Kroll, 1989). Figure 4 illustrates how the anions (A^-) and undissociated acids (HA) are distributed inside and outside the yeast cell for a given concentration of acetic acid and lactic acid at a particular pH of the medium. However, Narendranath *et al.* (2001b) found that acetic and lactic acids inhibit yeast growth by different mechanisms.

Therefore, the two major reasons for reduction in ethanol yield due to lactobacilli contamination

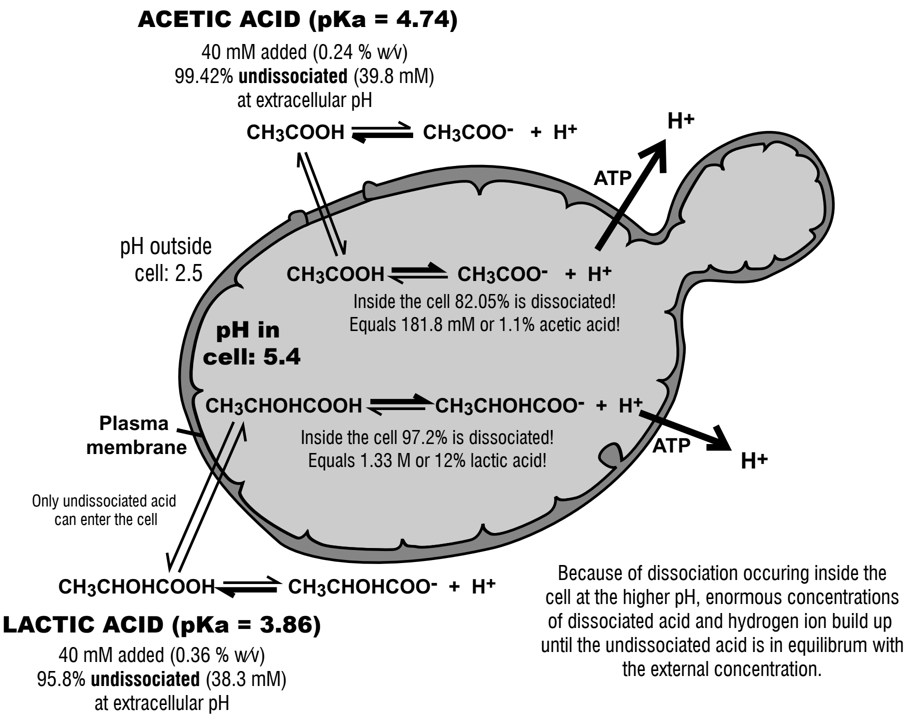

ACETIC ACID (pKa = 4.74)
40 mM added (0.24 % w/v)
99.42% undissociated (39.8 mM)
at extracellular pH
CH3COOH ⇌ CH3COO- + H+
H+
ATP
pH outside
cell: 2.5
CH3COOH ⇌ CH3COO- + H+
Inside the cell 82.05% is dissociated!
Equals 181.8 mM or 1.1% acetic acid!
pH in
cell: 5.4
CH3CHOHCOOH ⇌ CH3CHOHCOO- + H+
Plasma
membrane
Inside the cell 97.2% is dissociated!
Equals 1.33 M or 12% lactic acid!
ATP
H+
Only undissociated acid
can enter the cell
CH3CHOHCOOH ⇌ CH3CHOHCOO- + H+
LACTIC ACID (pKa = 3.86)
40 mM added (0.36 % w/v)
95.8% undissociated (38.3 mM)
at extracellular pH
Because of dissociation occuring inside the
cell at the higher pH, enormous concentrations
of dissociated acid and hydrogen ion build up
until the undissociated acid is in equilibrum with
the external concentration.

Figure 4. Illustration of the concentrations of anions and undissociated acids that would be present in the medium and inside the cell (based on the pH_{out} and pH_{in}) when 40 mM of either acetic acid or lactic acid is present. The concentrations were calculated based on the Henderson-Hasselbach equation. The concentrations differ based on the molar concentration of the acids in the medium and the pH_{out} and pH_{in} values. The concentrations indicated do not remain steady because there is constant pumping out of the excess protons (by H^+-ATPase) to raise the intracellular pH, resulting in further penetration of weak acid molecules into the cell that re-acidify the cytoplasm. In most situations, the pH of the medium also falls, affecting results. The concentrations of anions could reach 181.8 mM for acetate and 1.33 M for lactate under the stated conditions when 40 mM acid is added to the medium (adapted from Narendranath *et al.,* 2001b).

are: 1) Glucose that could be available for yeast to produce ethanol is taken up by these bacteria, and 2) the end products of metabolism, acetic acid and lactic acid, inhibit yeast growth and concomitantly ethanol production.

As little as 1% decrease in ethanol yield is highly significant to distillers of fuel alcohol (Makanjuola *et al.,* 1992). In large plants with outputs of 400 million to 1100 million liters of ethanol per year, such a decrease would reduce income by 1 to $3 million USD annually.

Management of lactobacilli in distilleries

Despite cleaning and sanitation procedures (covered in detail in elsewhere in this volume), bacteria may find their way into distillery processes. These bacteria must be removed as quickly as possible. The methods used in the ethanol industry to control contaminant bacteria include stringent cleaning and sanitation, acid washing of yeast destined for reuse (in case of breweries), adjustment of mash pH, and the use of antibiotics during fermentation. The method(s) used depends to a large degree on the end use of the alcohol.

USE OF ANTIBIOTICS

To control lactobacilli during fermentation, antibiotics are used in fuel ethanol plants. Antibiotics are compounds produced by microorganisms, which at low concentrations inhibit the growth of other microorganisms.

There are different kinds of antibiotics and they can be grouped according to their mechanism of action against bacteria.

Penicillin G has been widely studied (Day *et al.,* 1954) and used in the alcohol production industry during fermentation since the 1950s. Penicillin G is primarily active against Gram-positive bacteria. The extensive use of this antibiotic, which acts by inhibiting bacterial cell wall synthesis, has led to the emergence of resistant microflora. For this reason and due to the instability of penicillin G pH levels below 5, various other antimicrobials have been introduced or investigated for application including virginiamycin (Hynes *et al.,* 1997), streptomycin, tetracycline (Aquarone, 1960; Day *et al.,* 1954) and monensin (Stroppa *et al.,* 2000). Tetracycline is a broad spectrum antibiotic, where as penicillin V, monensin, and virginiamycin are more active against Gram-positive bacteria. The other antibiotics that have been tried such as streptomycin and polymixin are more active against Gram-negative bacteria. The typical usage rate for these antibiotics in the fermentation is 2-5 ppm. The mechanisms of action and the spectrum of these antibiotics are summarized in Table 2.

Although many antibiotics have been tested at a laboratory scale, the most widely used in the ethanol industry are penicillin G and virginiamycin. Antibiotic residues and establishment of antibiotic-resistant bacterial strains is a global issue, and one that ethanol producers must understand because of DDGS use in food animal diets. Penicillin G is inactivated during the alcohol fermentation (Islam *et al.,* 1999). Inactivation of penicillin G is significant at 35°C. At pH 4.8, the biological half-life of penicillin G is 24 hrs at 25°C and 4 hrs at 35°C, whereas at pH 3.8, it is <4 hrs for both temperatures. Moreover, any penicillin G still active at the end of fermentation is definitely destroyed in the still since penicillin G decomposes rapidly at temperatures over 52°C (Kheirolomoom *et al.,* 1999). In contrast, virginiamycin is not significantly altered during alcohol fermentation after 72 hrs at 35°C (Islam *et al.,* 1999). Research has shown that using over 2 ppm virginiamycin to control bacteria suppresses the rate of fermentation. Upon distillation (100°C for 30 min) virginiamycin activity is reduced to 2.6% of the original (Hamdy *et al.,* 1996). Residues can therefore be present in DDGS; which is a potential problem in markets where this antibiotic has been banned in animal feeds.

SYNERGY: ANTIBIOTIC COMBINATIONS

Using single antibiotics such as penicillin G or virginiamycin repeatedly can lead to development of resistance in microorganisms. Figure 5 shows the development of resistant

Table 2. List of some of the antibiotics used in the ethanol industry, their modes of action and activity spectrum.

Antibiotic	*Mechanism*	*Bactericidal/static*	*Spectrum*
1a. Penicillin G 1b. Penicillin V*	Inhibits cell wall synthesis	Bactericidal	Gram(+) bacteria
2. Bacitracin	Affects cell wall	Bactericidal	Gram(+) bacteria
3. Tetracycline	Protein synthesis inhibitor	Bacteriostatic	Gram(+) & Gram(-) bacteria
4. Streptomycin	Protein synthesis inhibitor	Bactericidal	Gram(+) & aerobic Gram(-) bacteria
5. Erythromycin	Protein synthesis inhibitor	Bacteriostatic, cidal at high doses	Gram(+) & Gram(-) bacteria
6. Polymixin	Affects cell membrane	Bactericidal	Gram(-) bacteria
7. Virginiamycin	Protein synthesis inhibitor	Bactericidal	Gram(+) bacteria
8. Monensin	Affects cell membrane	Bactericidal	Gram(+) bacteria
9. Chloramphenicol	Protein synthesis inhibitor	Bactericidal (or) static	Gram(+) & Gram(-) bacteria. Good against anaerobes. Heat stable?

* Has the same properties as penicillin G but is more stable at acidic pH.

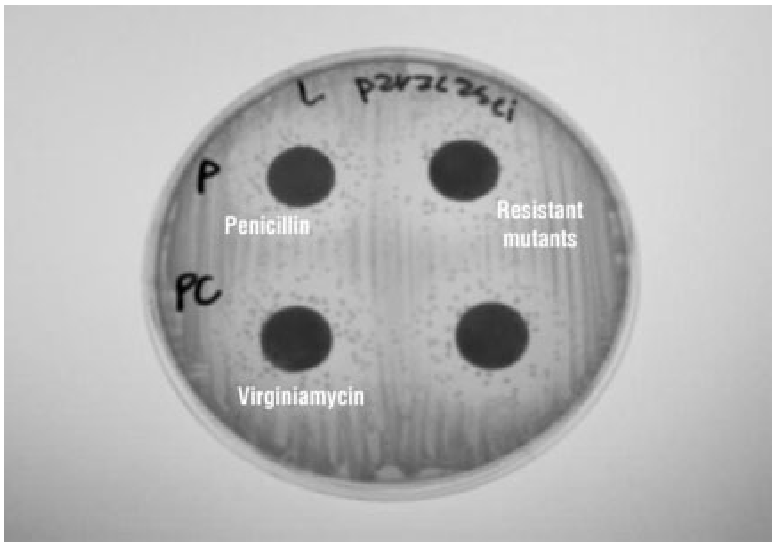


Figure 5. A typical disc assay performed to test the antimicrobial activity of penicillin G and virginiamycin against *Lactobacillus paracasei*. Note the growth of resistant mutants in the zone of inhibition.

mutants to single antibiotics such as penicillin or virginiamycin. One of the strategies to reduce the risk of resistance is to use a combination of antimicrobials, including non-antibiotic substances, thereby taking advantage of synergism in activity as well. Lactoside^{TM}, a proprietary blend of antimicrobials, reduced risk of resistance while increasing spectrum of activity and pH range. Following five years of use, no build up of resistance has been reported.

The advantage of balanced mixtures of antibiotics has been demonstrated in several practical situations. A recent experiment examined corn mash (30% dry solids; pH 5.6) contaminated with an aggressive *Lactobacillus paracasei* strain. Yeast was inoculated at 30 million cells/ml. Just prior to yeast inoculation, test fermentors were contaminated (inoculated) with *L. paracasei* at ~10^7 CFU/ml (in the treatments with bacteria). The antibiotics used, penicillin G, virginiamycin, and Lactoside™, were added from 10,000 ppm stock solutions. Virginiamycin was made up in HPLC-grade methanol. The temperature was maintained at 31.1°C (88°F) throughout the fermentation. Samples were withdrawn for analysis from each fermentor at 8, 24, 32, 48, 56, and 72 hrs. Viable cell counts were monitored by the spread plate technique. For enumeration of bacteria, the MRS agar plates (with 10 ppm cycloheximide) were incubated in anaerobic jars at 30°C. The plating was done in duplicate for each dilution. Results indicated that Lactoside^{TM} significantly reduced the viable numbers of bacteria. Virginiamycin was the least effective antibiotic at the dosage used in controlling *L. paracasei* (Figure 6).

In experiments performed to determine the minimum inhibitory concentrations (MIC) for various antibiotics against lactobacilli at various stages of their growth, Lactoside^{TM} at low concentrations killed *L. plantarum* at early, mid and late log phases of growth (Table 3).

Table 3. Minimum inhibitory concentrations for different antibiotics and Lactoside^{TM} (expressed in ppm) for *L. plantarum* at different stages of its growth.

Antibiotic	*MICs when antibiotic added at various stages of growth*			
	0 hrs	*Early log*	*Mid log*	*Late log*
Penicillin G	0.2	12.8	>12.8	>12.8
Penicillin V	0.2	12.8	>12.8	12.8
Virginiamycin	0.2	>12.8	>12.8	>12.8
Lactoside^{TM}	0.2	0.2	0.2	0.8
Oxytetracycline	>6.4	>6.4	>6.4	>6.4
Erythromycin	12.8	>12.8	>12.8	>12.8
Streptomycin	>12.8	>12.8	>12.8	>12.8

Similar results were obtained with four other species of lactobacilli. Figure 7 shows the regions of a typical growth curve for a microbe where the various antibiotics when used at low concentrations would be effective in killing the microbe. This information is important for a

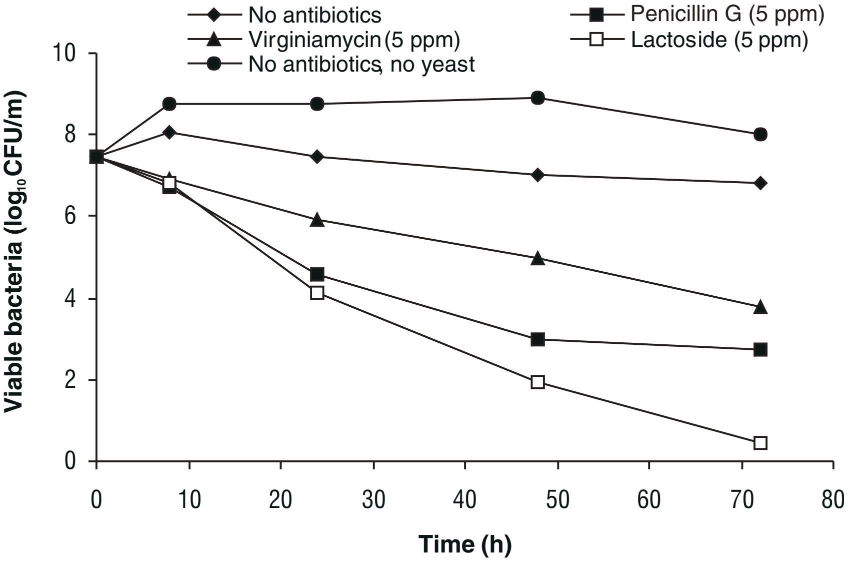


Figure 6. Survival of *L. paracasei* in the fermentation of corn mash (30% DS, pH 5.6) at 31.1°C (88°F) in the presence or absence of various antibiotics. Values are means of duplicate fermentations that had a CV of <8%.

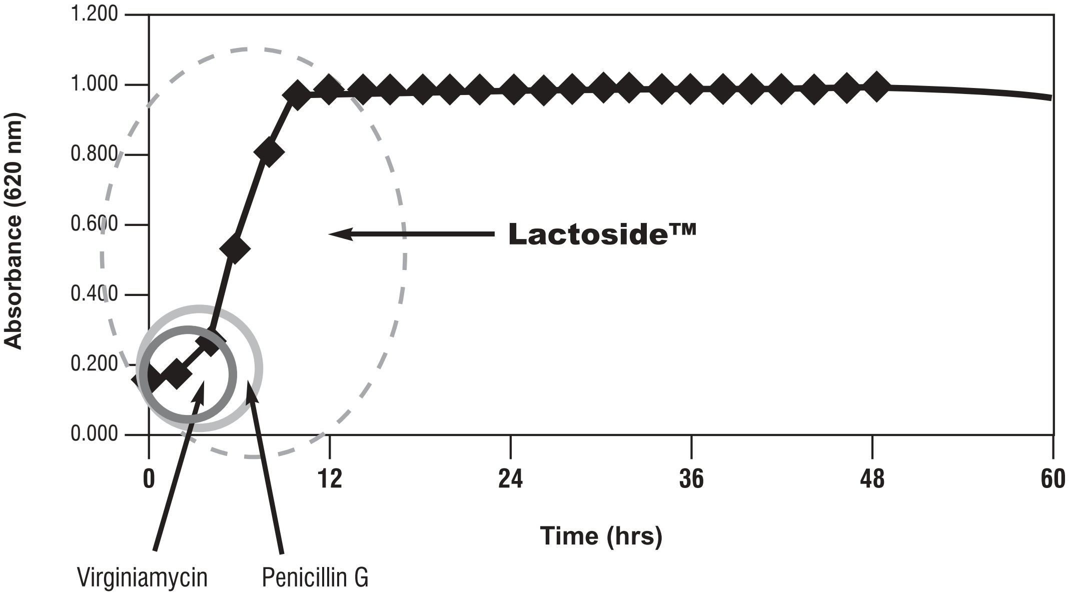


Figure 7. A typical growth curve of a microorganism showing at what stages antibiotics are effective at low concentrations.

distillery since contamination can be detected as early as 6-10 hrs from the start of filling a fermentor. By this time, the contaminants are approaching the mid-log phase of growth. Therefore, an antibiotic or antimicrobial effective at a low concentration at this stage would be an ideal choice.

The ideal antimicrobial should, however, possess all the following characteristics:

1. Broad-spectrum activity: effective against both Gram-positive and Gram-negative bacteria.
2. Effective over a wide pH range.

3. Control the bacteria at various growth stages.
4. Effective at low concentrations.
5. Bactericidal rather than bacteriostatic.
6. Able to be combined with other antibiotics or antimicrobials to avoid resistance development in bacteria.

FORMULATION OF ANTIBIOTIC COMBINATIONS

Factorial experiments are conducted to test interaction effects (either synergistic or antagonistic) among antibiotics used in combination to control growth of a test lactic acid bacteria. These experiments are conducted in corn mash rather than laboratory media so that the results can be translated to the field. From these experiments, the individual effects of the antibiotics can also be determined. Lactic acid is the parameter measured since lactic acid produced is directly proportional to the viable cell numbers of bacteria present in mash (Narendranath *et al.,* 1997). The results of the interactions are plotted in 3-D graphs with the two test antibiotics on x- and y-axes, and lactic acid on the z-axis. The graphs are created based on the response surface regression analysis of the data using the SAS program. Figure 8 shows the model created by using the equation based on the data obtained in an experiment. This equation can then be used to make formulations with correct proportions of each antibiotic. Experimental designs are available to generate such equations for studying the combination of more than two antibiotics.

It is becoming a common practice in some industrial operations to either under- or overdose antibiotics for controlling contaminating bacteria. Under-dosing antibiotics leads to the development of resistance in microorganisms. Overdosing antibiotics can affect fermentation rate by yeast (Hamdy *et al.,* 1996) and increase the chances that all the antibiotic will not be inactivated by the distillation process. Therefore, it is always advisable to use the antibiotics at the dosage recommended by the supplier/manufacturer.

LOWERING MASH pH CAUSES ETHANOL LOSS

Since the growth rate of lactic acid bacteria is reduced significantly at pH levels <5.0 (Kandler and Weiss, 1986), most ethanol plants tend to reduce the pH of the mash with sulfuric acid to <4.5 at the start of fermentation. Some continuous fermentation plants even start fermentations at pH of 4.0 or less. Lowering mash pH may reduce the growth rate of contaminant bacteria but it significantly reduces ethanol yield. In an experiment using mashes of various dissolved solids concentrations adjusted to pH values from 4 to 5.5, significantly higher ethanol was obtained when the initial

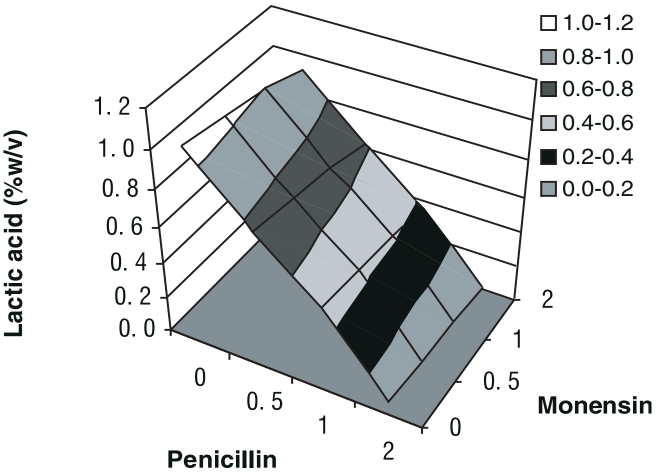


Figure 8. Lactic acid produced by *L. plantarum* in corn mash (pH 5.5, D.S. 24%) at 30°C as affected by various concentrations (ppm) of penicillin and monensin in combination in a 4 × 4 factorial experiment. Model was significant ($P<0.001$) with $R^2 = 0.9993$.

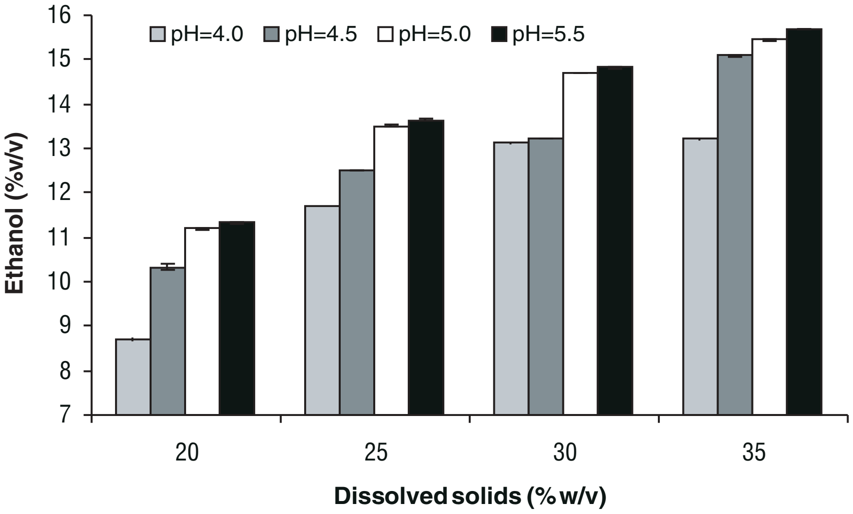


Figure 9. Ethanol produced by *S. cerevisiae* in 48 hrs at 30°C at different concentrations of dissolved solids in the medium at four pH levels. Coefficient of variation among the duplicates was <2%.

mash pH was 5.5 (Figure 9). Moreover, yeast can tolerate higher acetic acid (0.1-0.2%w/v) and lactic acid (up to 3%w/v) when the starting pH is 5.5, since at this pH these acids are primarily in the dissociated form. By the end of fermentation, however, the pH drops 1 unit to 4.5 under normal 'contaminant-free' conditions. Similar observations were also made with wheat and milo mashes.

The occurrence of bacterial contaminants in an industrial-scale ethanol production process is unavoidable, especially when the pH of the mash at set (start of fermentation) is between 5.0 and 5.5. Therefore, a good cleaning and sanitation regime combined with an effective antimicrobial program will reduce bacterial numbers and significantly increase ethanol yield, which ultimately results in increased profit for ethanol producers.

References

Alexander, M. 1971. Microbial ecology. John Wiley & Sons, Inc., London, UK.

Aquarone, E. 1960. Penicillin and tetracycline as contamination control agents in alcoholic fermentation of sugarcane molasses. Appl. Microbiol. 8:263-268.

Barbour, E.A. and F.G. Priest. 1988. Some effects of *Lactobacillus* contamination in scotch whisky fermentations. J. Inst. Brew. 94:89-92.

Booth, I.R. and R.G. Kroll. 1989. The preservation of foods by low pH. In: *Mechanisms of action of food preservation procedures* (G.W. Gould, ed). Elsevier, London, UK, pp. 119-160.

Bryan-Jones, G. 1975. Lactic acid bacteria in distillery fermentation. In: *Lactic acid bacteria in beverages and foods. Proceedings of the IV Long Ashton Symposium* (J.G. Carr, C.V. Cutting and G.C. Whiting, ed). Academic Press, London, UK, pp. 165-175.

Chin, P.M. and W.M. Ingledew. 1994. Effect of lactic acid bacteria on wheat mash fermentations prepared with laboratory backset. Enzyme Microb. Technol. 16:311-317.

Day, W.H., W.C. Serjak, J.R. Stratton and L. Stone. 1954. Antibiotics as contamination control agents in grain alcohol fermentations. J. Agric. Food Chem. 2:252-258.

Dolan, T.C.S. 1979. Bacteria in whisky production. Brewer 65:60-64.

Hamdy, M.K., R.T. Toledo, C.J. Shieh, M.A. Pfannenstiel and R. Wang. 1996. Effects of virginiamycin on fermentation rate by yeast. Biomass Bioenergy 11:1-9.

Hynes, S.H., D.M. Kjarsgaard, K.C. Thomas and W.M. Ingledew. 1997. Use of virginiamycin to control the growth of lactic acid bacteria during alcoholic fermentation. J. Ind. Microbiol. Biotechnol. 18:284-291.

Islam, M., R. Toledo and M.K. Hamdy. 1999. Stability of virginiamycin and penicillin during alcohol fermentation. Biomass Bioenergy 17:369-376.

Kandler, O. and N. Weiss. 1986. Regular, non sporing gram-positive rods. In: *Bergey's manual of systematic bacteriology*, Vol. 2 (P.H.A. Sneath, N.S. Mair, M.E. Sharpe and J.G. Holt, ed). The Williams & Wilkins Co., Baltimore, MD, pp. 1208-1234.

Kheirolomoom, A., A. Kazemi-Vaysari, M. Ardjmand and A. Baradar-Khoshfetrat. 1999. The combined effects of pH and temperature on penicillin G decomposition and its stability modeling. Process Biochem. 35:205-211.

Lindgren, S.E. and W.J. Dobrogosz. 1990. Antagonistic activities of lactic acid bacteria in food and feed fermentations. FEMS Microbiol. Rev. 87:149-164.

Maiorella, B., H.W. Blanch and C.R. Wilke. 1983. By-product inhibition effects on ethanolic fermentation by *Saccharomyces cerevisiae*. Biotechnol. Bioeng. 25:103-121.

Makanjuola, D.B., A. Tymon and D.G. Springham. 1992. Some effects of lactic acid bacteria on laboratory scale yeast fermentations. Enzyme Microb. Technol. 14:351-357.

Narendranath, N.V., S.H. Hynes, K.C. Thomas and W.M. Ingledew. 1997. Effects of lactobacilli on yeast-catalyzed ethanol fermentations. Appl. Environ. Microbiol. 63:4158-4163.

Narendranath, N.V., K.C. Thomas and W.M. Ingledew. 2000. Urea hydrogen peroxide reduces the numbers of lactobacilli, nourishes yeast and leaves no residues in the ethanol fermentation. Appl. Environ. Microbiol. 66:4187-4192.

Narendranath, N.V., K.C. Thomas and W.M. Ingledew. 2001a. Effects of acetic acid and lactic acid on the growth of *Saccharomyces cerevisiae* in a minimal medium. J. Ind. Microbiol. Biotechnol. 26:171-177.

Narendranath, N.V., K.C. Thomas and W.M. Ingledew. 2001b. Acetic acid and lactic acid inhibition of growth of *Saccharomyces cerevisiae* by different mechanisms. J. Am. Soc. Brew. Chem. 59:187-194.

Ralph, R.J. 1981. Practical aspects of operating an alcohol plant. In: *A Step to energy independence - a text book for fuel alcohol production* (T.P. Lyons, ed.). Alltech Technical Publications, Lexington, KY, pp. 255-265.

Salmond, C.V., R.G. Kroll and I.R. Booth. 1984. The effect of food preservatives on pH homeostasis in *Escherichia coli*. J. Gen. Microbiol. 130:2845-2850.

Stroppa, C.T., M.G.S. Andrietta, S.R. Andrietta, C. Steckelberg and G.E. Serra. 2000. Use of penicillin and monensin to control bacterial contamination of Brazilian alcohol fermentations. Int. Sugar J. 102:78-82.

Chapter 21

Managing the Four Ts of cleaning and sanitizing: time, temperature, titration and turbulence

JIM LARSON AND JOE POWER

North American Biosciences Center, Alltech Inc., Nicholasville, Kentucky, USA

Introduction

Cleaning and sanitizing is an integral part of the operation in many processing facilities including distilleries, ethanol plants and breweries. The cleaning and sanitizing effort represents a significant investment of time, labor and operating costs. At the same time there is usually a positive payback for this effort in terms of yields, efficiencies, safety and product quality.

In a clean plant that is running efficiently with no infection problems, there is always the temptation to reduce operating costs by trimming back cleaning times and chemical usages. This type of ongoing evaluation is a worthwhile part of plant management. We should always strive to clean and sanitize to the extent required by the process and the equipment but overkill in this area should be avoided. If a little bit is good, a lot is not necessarily better.

To achieve the proper degree of cleaning and sanitizing at an acceptable cost, there are a myriad of approaches and tools that can be used. Sometimes called the ‘cleaner’s bag of tricks’, these tools can be grouped in four categories known as the ‘Four Ts’ of Cleaning and Sanitizing. The Four Ts are:

- **Time**
- **Temperature**
- **Titrations**
- **Turbulence**

Time refers to the frequency of running the cleaning operation, for example weekly, daily, or after each batch. In a given cleaning cycle it also refers to the length of each step. For example a 5 min rinse, a 1 hr caustic wash, etc.

Temperature refers to the temperature of the cleaning solutions used in each step of the cleaning cycle. The first rinse may be at ambient temperature while the caustic wash may be specified between 150 and 160°F.

Titrations refers to the chemistry of the cleaning solutions. This includes selecting the right cleaning chemical for the job being done. It also includes the concentration of chemicals used in the cleaning solutions.

Turbulence is the mechanical action in the cleaning program that will physically scrub soil from dirty surfaces. Turbulence encompasses the use of scrub brushes and high-pressure nozzles as well as pumping cleaning solutions at high velocities through dirty pipelines.

For any cleaning job, many combinations of time, temperature, titrations and turbulence can be used to achieve effective cleaning at a reasonable cost. There are also many combinations that will not work because of a deficiency in one or more of the Four Ts. For example, cleaning time may be too short or too infrequent. The detergent may be too weak, it may be at too low a temperature or it may not be the right choice for the soil type present (Figures 1 and 2).

If cleaning is inadequate, it is usually possible to correct it by increasing the intensity of one of the Four Ts (Figure 3). If the turbulence created by the velocity of cleaning solution flowing in a pipeline is inadequate, it may be possible to compensate by cleaning for a longer time, by increasing the concentration of the cleaning chemicals or by raising the cleaning solution temperature.

Our challenge is to achieve an acceptable degree of cleaning and sanitizing in specific processes. We sometimes go through elaborate procedures but in the end still have infections. Why?

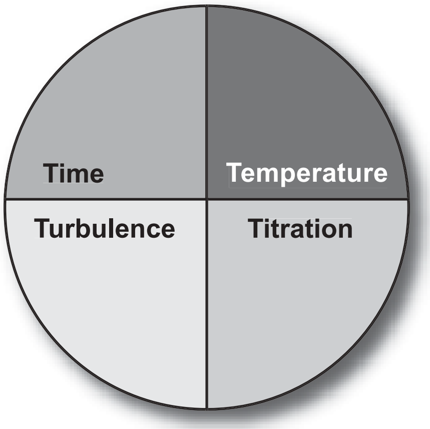


Figure 1. Some combinations work.

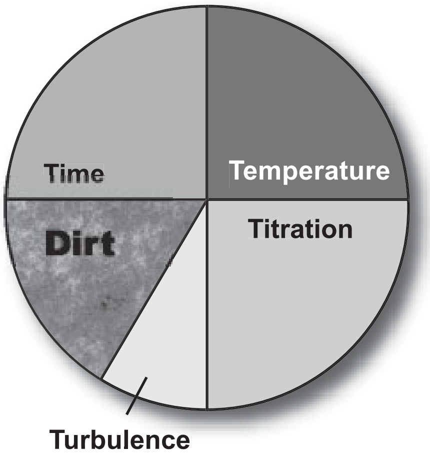


Figure 2. Some combinations do not work; and dirt remains.

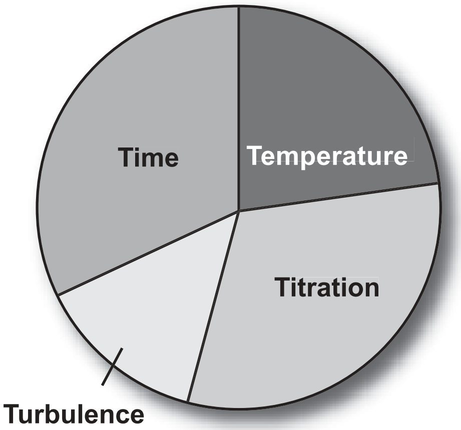


Figure 3. Deficiencies in one of the Four Ts can be corrected or compensated by changing another.

This paper examines the tools at our disposal for effective cleaning and sanitizing – our cleaning bag of tricks. Proper attention to the Four Ts in the toolkit and how they are combined is needed to make cleaning and sanitizing effective.

Definitions

Cleaning is reducing the amount of soil to an acceptable level. Cleaning is usually done with a combination of water and added detergents. Undercleaning means that we have not done the job properly. Gross overcleaning means wasted resources – time, chemicals and manpower. An acceptable level of cleaning in a pharmaceutical plant would probably be overcleaning in an ethanol distillery.

Sanitizing is reducing the population of viable organisms on a clean surface to an acceptable level. Again, 'acceptable' has different meanings in different operations. Within the ethanol industry it may even vary from plant to plant. Heat and/or sanitizing chemicals are used for this step.

Disinfection is the destruction of all vegetative microorganisms, but not necessarily spores.

Sterilization is the complete destruction of all organisms including spores and viruses.

Soil is any unwanted material left on a surface that needs to be clean. Soils can be composed of sugars, salts, fats, proteins, microbes, scales and other mineral deposits. Knowing the nature of the soil is necessary when planning the cleaning procedure used to remove it. Soils left on surfaces harbor contaminating bacteria, reduce efficiency in heat exchangers and plug pipes and other passageways.

CIP is the acronym for cleaning-in-place. Pipelines, tanks, process equipment and accessories are cleaned by pumping cleaning solutions through them without disassembly or manual cleaning. CIP processes are usually automated.

COP is cleaning-out-of-place. COP involves manual disassembly and cleaning by hand.

Therefore, cleaning is defined as the removal of soil while sanitizing is the reduction of viable organisms remaining on the clean surface. An important concept in cleaning and sanitizing is that first we remove the dirt, *then* we sanitize. *Sanitizing dirt is not in the program!*

Time

If you don't get it clean the first time, wash it again. Spend more time cleaning and you will get better results. This sounds like a simple solution, but a dilemma arises because time spent cleaning could be time spent producing product.

Figure 4 illustrates this in the operation of a batch ethanol fermentor. In the fermentation cycle, a period of time is set aside for cleaning and sanitizing between batches. The ethanol concentration curve, however, shows that we can produce more ethanol in the fermentor if we omit all or part of the cleaning time. But what will the longer-term results be if we take shortcuts in cleaning? We must find a happy medium, which is the optimum amount of time taken from production to keep the process equipment clean. The other three Ts of cleaning and sanitizing can help us do this.

Time refers to the frequency of cleaning as well as the length of the cleaning cycle. We learn from experience that some parts of the process are very sensitive to infection and require frequent cleaning. An example is the yeast propagation system, which is thoroughly cleaned and sanitized after every use. Other parts such as the slurry tank or liquefaction tank are less critical and may be cleaned every 1-6 months.

Continuous fermentation systems present special problems compared to batch fermentors. A continuous fermentation train may be shut down only one or two times a year for cleaning. This shutdown requires a total stoppage of production, so it has significant cost implications. Because of the continuous flow through the fermentation train, there is some degree of flushing of the infecting organism. Frequently the culture yeast grows more slowly than the contaminant, and reduced ethanol yield requires some action. Antibiotics such as Lactoside ™ and Allpen™ and antimicrobials may be added to the mash to kill bacteria without requiring a shutdown. For a wild yeast infection it is usually

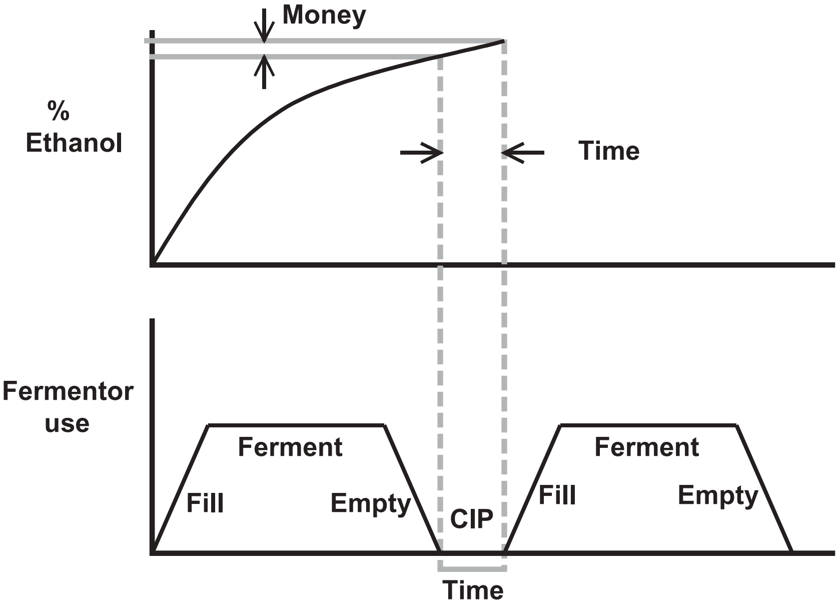


Figure 4. The dilemma between cleaning time *vs*. production: time = money!

necessary to shut down and clean because agents that kill the wild yeast will also kill the culture yeast.

Organizing a cleaning schedule for every piece of equipment in the plant is an important part of the CIP program. An example of such a schedule is shown in Table 1.

Temperature

Warm cleaning is more effective than cold cleaning. Within limits, this is usually true in cleaning and may or may not be true in sanitizing. Caustic soda (sodium hydroxide) cleaning solutions can be used at ambient temperatures but they are normally heated, as high as 82°C (180°F). Reactions proceed faster at higher temperatures and many soils are more soluble in hot water than in cold water. The cleaning process can therefore be faster and more effective if hot solutions are used.

Table 2 shows the results of a cleaning study comparing warm and cold detergent solutions to clean dirty beer lines. The amount of soil present was measured with an ATP-detecting luminometer. Starting from a 'soil overload' condition (>500,000), the cold solution left 300 times as much soil as the warm solution.

Table 2. Effect of temperature on amount of soil present before and after use of cold or warm cleaning solutions.

	Cold cleaning 23°C (74°F)	*Warm cleaning 57°C (135°F)*
Before cleaning	>500,000	>500,000
After cleaning	18,242	59

When raising the temperature of a cleaning solution it is important to check the chemical compatibility of the equipment material of construction with the hot cleaning solution. Some hot acid solutions, for example, may be too corrosive for metals such as mild steel or brass.

Most sanitizing solutions lose effectiveness when the temperature is raised above a threshold level. For example, cool or tepid water should be used for preparing solutions of Iotech™ and other iodophor sanitizers and they should be stored below 38°C (100°F). At higher temperatures the vapor pressure of the active ingredient causes it to vaporize.

Temperature can be an effective tool in the 'cleaning bag of tricks.' For example, if we want to reduce the time spent cleaning a product transfer line without sacrificing cleaning effectiveness, a

Table 1. Control room CIP schedule.

Monday		*Tuesday*		*Wednesday*		*Thursday*		*Friday*		*Saturday*		*Sunday*	
DAYTIME	*Start time*	*DAYTIME*	*Start time*	*DAYTIME*	*Start time*	*DAYTIME*	*Start time*	*DAYTIME*	*Start time*	*DAYTIME*	*Start time*	*DAYTIME*	*Start time*
Distillation: Spiral heater:		Distillation: Spiral heater:		Distillation: Spiral heater:		Distillation: Spiral heater:		Distillation: Spiral heater:		Distillation: Spiral heater:		Distillation: Spiral heater:	
NIGHT		*NIGHT*		*NIGHT*		*NIGHT*		*NIGHT*		*NIGHT*		*NIGHT*	
Exch:1302 A or B Exch. 1303 Exch 1302C		Exch: 1400 Exch: 1401		Exch: 1402 Exch: 1403		Exch: 1404 Exch: 1301B Exch: 1406		Exch: 1302 A or B Exch: 1302C Exch: 103		Evaporator			

warmer or hotter cleaning solution could be the answer.

Titrations

Titrations, the third T in the cleaning and sanitizing bag of tricks, refers to the chemistry of cleaning and sanitizing chemicals and solutions. It includes matching the type of chemical cleaner to the type of soil present and using that chemical at a strength that is both effective and affordable. It is therefore essential to have as much information as possible about the soil we are trying to remove (McCabe, 1999; Kretsch, 1994).

THE NATURE OF SOILS

Any of the compounds present in raw materials or in the water supply used for fermentation may become insoluble and be deposited on equipment surfaces as an unwanted soil. Methods used to remove these soils depend on the specific nature of the material involved.

Carbohydrates

The soluble sugars present during fermentation may be left on surfaces, especially the non-fermentable sugars. A much bigger problem is the higher molecular weight carbohydrates such as gums, which can form tough deposits on surfaces. Retrograded starch can form when starch is not sufficiently digested by α-amylase enzymes. Depending on the type of grains used, gums such as ß-glucans or pentosans may become soluble in the mash and then be deposited later on. Dextrans produced by *Leuconostoc* bacteria from sucrose can be produced in plants fermenting sucrose-containing materials.

Proteins

Most of the protein present in grains is insoluble and passes through mashing and fermentation in the insoluble form. The soluble portion of the protein may become denatured by conditions such as temperature changes or pH shifts. As the protein becomes insoluble it may be deposited onto various surfaces. If tannins are present, they may react with proteins in a reaction similar to the tanning of leather, forming tough, water-resistant deposits.

Oils

Oils are liquid fats and are intrinsically insoluble. Much of the oil present in grains is broken into microscopic droplets or emulsions that are suspended in the mash. As conditions change, the emulsions may coalesce forming larger deposits of insoluble oil.

Inorganic compounds

Various inorganic compounds may become insoluble during mashing or fermentation. Most commonly calcium in water reacts with the oxalate present in grains to form insoluble calcium oxalate, commonly called beerstone. Carbonates present in water may combine with calcium to form calcium carbonate scale. Magnesium hydroxide tends to co-precipitate as part of the carbonate scale. Occasionally water with high silicate content may generate a silica scale as the pH drops. Soluble silicate is converted by acid to insoluble silica (SiO_2).

Biological deposits

Many microorganisms tend to grow better on surfaces than when freely suspended in a liquid. The layer of microorganisms that forms together with other materials such as non-living soil and external polysaccharides produced by the microorganisms is called a *biofilm* (Czechowski and Banner, 1992). A biofilm may be observed as a slimy layer on tanks used to store water. Fortunately, most microorganisms found in fermentation do not have a pronounced tendency to form a biofilm, but films of yeast or bacteria growing inside a surface scale are frequently observed. These films are a potent source of contamination.

MATERIALS USED FOR CLEANING

Water

Water is a good solvent for many compounds, especially polar compounds. Materials such as simple sugars, soluble proteins and the soluble products of fermentation are easily removed with water. Most compounds are more soluble in hot water than cold, so heating water helps make it a better cleaner. Exceptions are the water hardness compounds, particularly those of temporary hardness. These become less soluble at higher temperature leading to the deposition of calcium carbonate-based water scales. An important consideration in the use of hot water for cleaning is to prevent formation of scales by softening the water. Many cleaners contain water-softening ingredients.

Pure water has a relatively high surface tension, as shown by the fact that water tends to form round drops when placed on a surface. Water is described as not being good at wetting because the water forms droplets and does not wet the entire surface. Compounds that concentrate at the surface of water often decrease the surface tension of water droplets, making it easier for the water to spread out and cover more of the surface. These compounds are called wetting agents.

Alkali

An alkali is something that raises the pH of water. The pH depends on the concentration of hydroxide ions: the more hydroxide the higher the pH. The most common alkali used for cleaning is caustic soda (sodium hydroxide), which is an excellent source of hydroxide ions and is relatively inexpensive. Unfortunately, caustic soda has poor rinsability; it does not rinse away very easily after it is used for cleaning. Potassium hydroxide is also a strong alkali and it rinses more easily than sodium hydroxide, but it is significantly more expensive. Addition of surfactants to cleaning compounds with caustic soda will improve rinsability.

Other alkaline compounds include silicates, phosphates and carbonates. Sodium metasilicate is a commonly used alkali. It provides a high pH, although not as high as caustic. It tends to keep removed soil in suspension, providing good removal from the area cleaned. Silicate does not tend to corrode soft metals such as aluminum and copper, while caustic attacks these metals. Silicates are also less dangerous to handle.

Alkaline phosphates, especially trisodium phosphate, provide significant alkalinity, but less than caustic or silicates. It is safe for use in hand cleaning applications. It helps to soften water by forming an easily removed precipitate with hardness compounds. It can be combined with sodium hypochlorite to form a co-crystal, which is a convenient, stable source of chlorine.

Sodium carbonate or washing soda is a mildly alkaline compound that helps soften water as well as provide alkalinity. Its main use is in a mixture with solid caustic soda where its anti-caking activity helps prevent handling problems.

When alkaline compounds raise the pH level, they increase the amount of negative hydroxide ions present. This has two main effects. Bonds between the amino acids of proteins can be hydrolyzed or broken by reaction with hydroxide. This makes the proteins smaller and more soluble so that they can be removed. Acid compounds can have their acidic hydrogen removed by neutralization with hydroxide. The compounds are converted to their negatively charged ionic form. The negative ions repel each other, leading to a breakup of large aggregates of soil to smaller ones. This breakdown is called *peptizing*. The negative ions are also more soluble in water, leading to their removal from the surface. Proteins, composed of amino acids, are very effectively cleaned by alkali. If the alkali is strong enough, carbohydrates can also be converted into negative ions leading to their easier removal.

Alkali will also break down fats and oils to their component glycerol and free fatty acids. This is the basis for making soap from fat and alkali, and the process is called *saponification*. Provided that hardness ions are not present to precipitate them and that the high pH keeps the fatty acids in the ionized form, fats and oils are effectively removed by alkali.

Most inorganic scales are not effectively removed by alkali.

Chelating agents and sequestrants

Chelation is the binding of ions, most importantly the hardness ions calcium and magnesium, so that the hardness does not cause precipitation and scale formation. Common chelating agents are EDTA (ethylene diamine tetraacetic acid) and NTA (nitrilo triacetic acid). Sequestrants, especially the polyphosphates, have a similar action. Sodium gluconate acts as a chelator under strongly alkaline conditions and is often combined with caustic soda.

A strong chelating agent can also help dissolve inorganic scale that has already formed on surfaces. Chelators such as EDTA are relatively expensive and relatively large amounts are needed to remove scale where it has acumulated to significant depths. It is a good idea to measure the amount of free EDTA remaining in solution when it is being used for scale removal to make sure that not all of the compound has formed complexes with hardness ions.

Manufacturers of EDTA do not recommend using EDTA at high pH such as a mixture with caustic soda. The reason is because it loses its effectiveness at chelating magnesium at the high pH, but its chelation of calcium is not affected. Since cleaning difficulties are more associated with calcium than with magnesium, this caution can often be ignored.

Surfactants

Surfactants concentrate at the surface of water. They have both hydrophobic (water-fearing) and hydrophilic (water-loving) properties. Both properties can be satisfied at the same time at the surface where the hydrophilic portion can remain in the water and the hydrophobic portion can exit the water. The surface of the water is stabilized by surfactants. This means that water can more easily spread out and wet larger areas of surface. By increasing the wetting ability of cleaning solutions, surfactants allow the cleaner to spread out and clean more of the surface. By a similar mechanism it also helps the cleaner penetrate small crevices in the soil, increasing its effectiveness.

Surfactants may also congregate at the interface between water and small particles of soil. They can form a stabilizing sphere around the particle that keeps it in suspension and allows it to be efficiently swept away from the surface. Particles stabilized in this way are called *micelles*.

The oldest surfactant used for cleaning is soap. Soap is free fatty acid. As a rather insoluble acid, it only remains soluble if the pH is high (alkaline), stabilizing the acid in water. Soaps also form insoluble salts with the hardness ions, calcium and magnesium (bathtub ring) so they work poorly in hard water.

Newer synthetic detergents such as alkyl (from oil) benzene sulfonates overcome the problems of soap. The sulfonic acid portion is more easily ionized, so the pH does not need to be so high. The acid also does not tend to form salts with water hardness, so these detergents can be used in hard water. The drawback with these synthetic compounds, called anionic detergents, is that they are so surface active that they form a heavy layer of foam when sprayed onto surfaces in clean-in-place applications. Their use is therefore limited in industrial cleaning.

Another class of synthetic detergents, non-ionic detergents, does not have a strong tendency to form foam. They therefore can be used in clean-in-place applications. Most of these are polyoxyethylene compounds.

Acids

Acids are used in cleaning especially where removal of inorganic scales is necessary. Calcium compounds do not dissolve well in alkali, but are often quite soluble in acid. Most commonly, phosphoric acid is used or a combination of phosphoric and nitric acids. Sulfamic acid is also occasionally used.

An acid cleaning can follow an initial cleaning with a caustic soda-based alkaline detergent, which removes the organic components of the soil. The acid is then used to remove inorganic residue.

Broader range detergents based on acid with additional ingredients to remove organic soils have been used in some applications, but their use is not widespread.

Bleach

Oxidizing bleaches are used to partially oxidize components of the soil. Bleach can oxidize subunits of long chain proteins or polysaccharides, producing smaller and more soluble fragments, which can be more easily removed. Bleaches are usually added to other components of a formulated detergent to enhance overall cleaning.

Chlorine is the most commonly used bleach. Sodium hypochlorite, produced by adding chlorine to a caustic solution, can be added in small amounts to alkaline cleaners, such as caustic soda. Chlorinated trisodium phosphate, a stable solid, is also commonly used.

Typically about 200 ppm of chlorine equivalent is present in the cleaning solution.

Chlorine can be corrosive to stainless steel if left in contact for long periods of time, so the equipment should be thoroughly rinsed immediately after cleaning. Chlorinated alkali cleaners are very effective at removing soils with high protein content and also beerstone scales. Probably the beerstone is stabilized by organic compounds between the calcium oxalate crystals, and the cleaner removes the organic part very efficiently, making it easier to remove the exposed crystals.

The chlorine in cleaners is diluted to a much lower concentration than in the original sodium hypochlorite; and diluted hypochlorite is much more stable than concentrated. Diluted cleaners have been used at temperatures as high as 80ºC (176°F).

Hydrogen peroxide is also effective as a 'bleach' for cleaning. Stabilized peroxides such as sodium percarbonate and sodium perborate are available in powdered form. These are added to cleaner or blended with solid cleaning products.

SANITIZERS

The use of a sanitizer after effective cleaning can provide nearly sterile equipment. It should be emphasized that the cleaning must be extremely thorough in order to achieve this level of sanitation. Cleaning removes most dirt from surfaces and a part of this soil will be the microorganisms left behind after previous use. Most of the removal of microorganisms will occur during the cleaning step. Sanitizers remove most of the last organisms remaining behind.

Chlorine-releasing materials

The most commonly used 'chlorine' solution is actually a solution of sodium hypochlorite. Adding chlorine gas to a caustic solution produces sodium hypochlorite, and under the right conditions the chlorine is slowly released to do its sanitizing job. The sodium hypochlorite can exist in two forms, hypochlorite ion and hypochlorous acid. It is the hypochlorous acid that attacks microorganisms and provides the sanitizing effect. When the sodium hypochlorite is mixed with water, the pH of the solution decreases and hypochlorous acid begins to form. The lower the pH, the more hypochlorous acid is formed and the more effective sanitation will be. Unfortunately, if the pH drops too low, free chlorine gas begins to be released, leading to a very dangerous situation. Therefore, for effective use, the pH should be below 8.2 but not lower than 7.0. There is a very narrow range where hypochlorite should be used.

Dual halogen sanitizers have been developed that extend the effective range of chlorine-based sanitizers. A source of bromine is added to hypochlorite, allowing hypobromite to be formed. The dual halogens are effective at about one pH unit higher than plain hypochlorite.

It may seem paradoxical that hypochlorite as a bleach can be used in high pH alkaline cleaning formulations, but that it should be used at nearly neutral pH as a sanitizer. The bleaching action is indiscriminate, while the antimicrobial action is more specific, appearing to depend on the ability of the non-ionized form to penetrate and exert killing action inside living cells.

Chlorine in the form of hypochlorite is the least expensive sanitizer available. It is effective against a wide range of microorganisms, including spores. Its action is not diminished in hard water. If residual soil is present, the hypochlorite will bleach or oxidize the soil and be lost. It is important that equipment be thoroughly cleaned before a chlorine sanitizer is used or it will be inactivated before it can exert its sanitizing effect. Chlorine is also corrosive to many metals. It may be used on stainless steel at low concentrations, but it must be thoroughly removed by rinsing after use.

Chlorine dioxide

Chlorine dioxide (ClO_2), is a relatively new sanitizer based on chlorine. Chlorine dioxide will kill organisms more quickly and at lower concentrations than hypochlorite. Typical use rates are 2 to 10 ppm, measured as available chlorine, versus 25 to 200 ppm for hypochlorite. When residual soil is present, chlorine dioxide reacts more slowly than does hypochlorite so the need for thorough cleaning before use is reduced. It also can be used over a wide pH range since it dissolves as a gas, not forming an acid or salt in water. It is less corrosive than hypochlorite. The biggest disadvantage of chlorine dioxide is that it is relatively expensive.

It is a gas and will not remain in water solution for long periods of time; it must be generated and used on the same day. Many methods of generation are available. Acid conversion of chlorite is used on a small scale, but is inefficient. Larger scale generators may use dangerous chemicals such as chlorine gas or hydrochloric acid. Electrolytic generators are a newer development that promise greater efficiency and safety.

Iodine-releasing materials

Iodine is a very efficient sanitizer when used at a concentration of about 25 ppm at colder temperatures, and at an acid pH. Iodine tinctures in water are not very stable and actively stain materials with which they come into contact. Industrial use of iodine is usually in the form of a complex with detergents and acid called an *iodophor*. When concentrated iodophor is diluted in water, the iodine is freed from its complex with detergent. The acid in the iodophor reduces the pH of the diluted solution to the acid range where iodine is most effective. The acid can also help dissolve mineral scale left behind after alkaline cleaning. Because of the detergents in the iodophor, the sanitizer tends to foam when used in spray applications. The recent development of iodine stabilized with chlorine, 'stabilized iodine', has eliminated foaming as a problem and requires about half as much iodine to do the same sanitizing job. Since stabilized iodine is relatively new, it has not been legally cleared for use as a food equipment sanitizer in all areas.

Iodine must be used at an acid pH to be most effective. If an iodophor is used at higher than recommended dilution, the pH of the mixed sanitizer should be checked to make sure that the pH of the diluted solution is not too high. Since iodine tends to stain, be certain that staining can be tolerated before using an iodine based compound. There is little tendency to corrode metal equipment unless the metal is not resistant to the acid contained in iodophors.

Anionic surfactants

The anionic surfactants are not very commonly used as sanitizers, but they do have properties that make them the choice for some special applications. They are surface-active compounds and are active at low pH, so they must be combined with acid. While hypochlorites, iodophores and chlorine dioxide all dissipate or evaporate in a relatively short period of time, anionics are completely stable. They will last for weeks or months and are therefore the best choice for applications where equipment is to be stored in water or filled with water for longer than a few days. The practice of storing equipment soaking in plain water is extremely unsanitary and is a very common but bad practice. Bacteria will grow in the water, especially if some residue is left on the equipment. Slimy layers that often form on the equipment and storage containers are layers of billions of bacteria or a biofilm. Store the equipment with mild acid and an anionic surfactant and it will remain clean for months.

Being detergents, some of the anionic surfactants are also fairly good cleaning compounds and acid cleaning can also be accomplished with some of these compounds.

Peracetic acid

Peracetic acid is a complex of acetic acid with hydrogen peroxide. The peroxide is the sanitizer. It is effective at about 200 ppm of peroxide and at colder temperatures. It is expensive and is used mainly in special situations where residue of chloride or iodide from less expensive sanitizers or some of their reaction products cannot be tolerated, especially some food applications.

Quarternary ammonium compounds

Quarternary ammonium compounds, called 'quats', are stable, effective and long lasting, tending to leave a residue. They adsorb to surfaces and have a residual killing effect for relatively long periods of time. Usually the residue is not desirable in vessels such as fermenting tanks since the residue will also kill some yeast. If a lot of yeast are present, the number killed may not be too significant, so quats are occasionally used to sanitize these vessels. A better application is to use quats on areas where yeast should not be present. They are used on the outside of fermenting tanks and on floors where spills are likely to occur to keep down the growth of undesirable organisms such as molds on these surfaces.

Hot water

Hot water, over 180ºF (82°C), is a very effective sanitizer because the heat can be transferred to areas that are not penetrated by chemical sanitizers. Cost of fuel to generate hot water is a factor that limits its use as a sanitizer.

Hot caustic

Caustic (NaOH) by itself is not a sanitizer. Bacteria are often found in high numbers in caustic solutions stored at room temperature. Hot caustic, though, will very effectively kill microorganisms. The temperature must be at least 110ºF and temperatures above 130ºF give good sanitizing in a matter of a few minutes. The exact effect depends on the time, temperature and caustic concentration used. Many areas have legal requirements for washing and sanitizing reusable containers with caustic solutions.

Turbulence

Turbulence refers to the scrubbing and scouring action of cleaning solutions as they flow through pipelines or onto the dirty surfaces inside tanks and other equipment. More turbulence improves cleaning. Sometimes the issue is more basic than the degree of turbulence: does the cleaning solution even contact the soil? Large diameter horizontal pipes will not completely fill with liquid if the flow rate is too low. 'Shadow areas' can also prevent cleaning solution from contacting the soil.

For pipelines, effective cleaning requires velocities of 1-1.5 to 3 m/s (5-10 ft/s). We are more used to thinking in terms of flow rates like cubic meters per hour or gallons per minute. This requirement means that bigger pipes need higher flow rates for cleaning. Table 3 shows the flow rates required for 5 ft/s (1.5 m/s) in pipes with diameters from 75 mm to 300 mm. It is important to note that when the pipe diameter is doubled, the required flow rate for cleaning must be *four* times as large. This has a large effect on the size of pumps needed for CIP.

Table 3. Turbulence in pipelines: flow rates required for effective cleaning as pipe diameter increases[1].

Pipe size		*Flow rate for 5 ft/sec (1.5 m/sec)*	
Inches	*Millimeters*	*US gpm*	*Liter/min*
3	75	102	386
4	100	182	689
6	150	410	1552
8	200	728	2755
12	300	1620	6130

[1] Anon (1992).

A rule of thumb for cleaning process equipment (e.g., a heat exchanger) is that the cleaning solution flow rate should be 1.5 times the normal process flow rate to achieve good turbulence. An effective trick is to clean the equipment in both the forward-flow and the reverse-flow directions (Figure 5).

SANITARY DESIGN

One of the greatest impediments to achieving turbulence in cleaning is the presence of dead ends in process pipelines and other equipment (Peter, 1983). A dead end can be a length of pipe that is wetted by the cleaning solution but without sufficient velocity to clean the surfaces. A common example is the bypass line, shown in Figure 6. When the heat exchanger is cleaned, solutions cannot flow through the dirty bypass unless someone opens the bypass valve. This may or may not happen. It should also be noted that when the bypass valve is open, the total solution flow is split in two directions: through the equipment and through the bypass line. The flow rate through each is therefore reduced and the velocity of the cleaning solution is compromised.

Figure 7 shows some design tricks that can eliminate the creation of dead ends before they are built into the system. For example, branch lines from the main line should have the valve installed not more than one diameter from the wall of the main pipeline. This ensures turbulence in the branch – that it will not be a dead end for cleaning. Tees in the main line should be manufactured tees made with smooth rounded contours that are sanitary and cleanable.

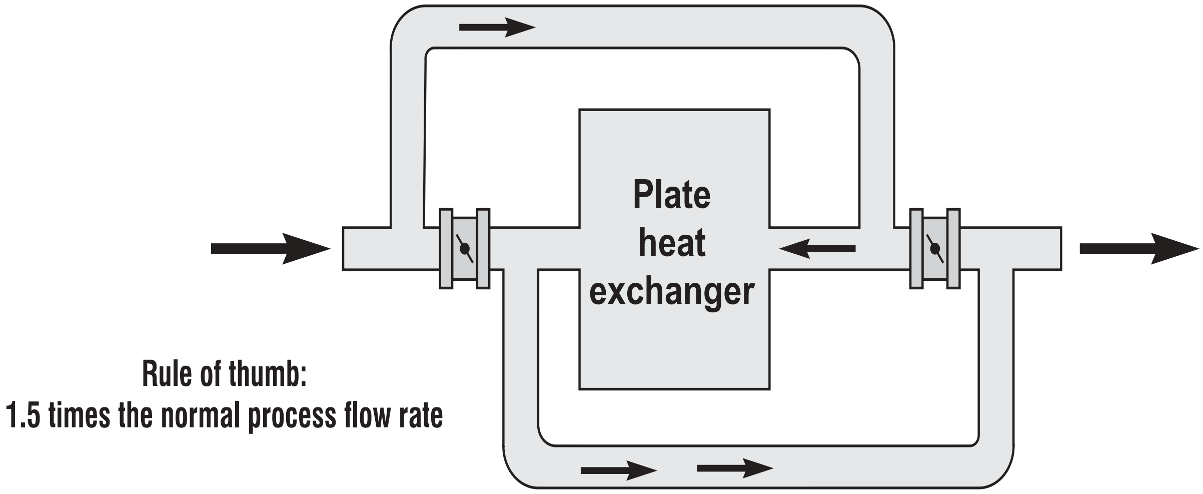


Figure 5. Turbulence in process equipment.

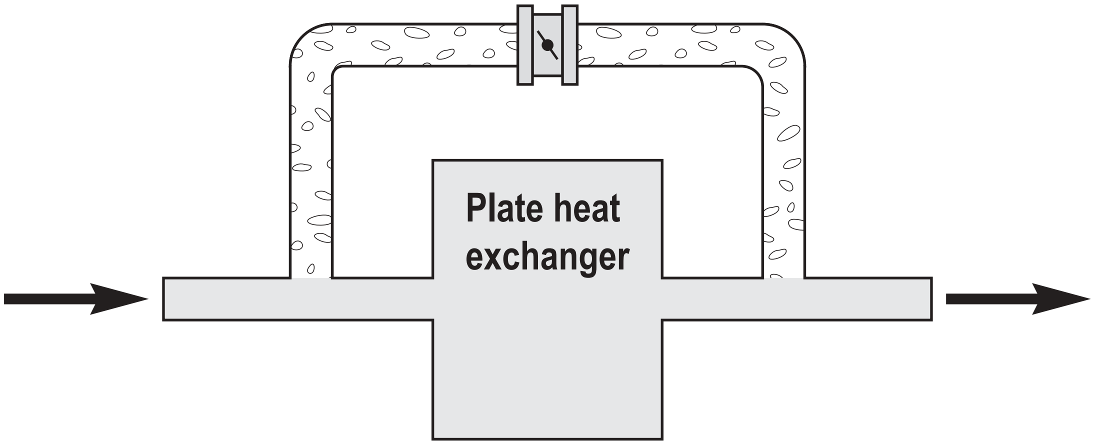


Figure 6. Dead ends and turbulence: did the bypass get cleaned?

Fitted joints, that is homemade or fabricated-in-the-field tees, are not sanitary and should not be accepted.

Figure 8 shows an innocent looking tee with a valve on the branch line. This section of pipe was opened for inspection and the branch pipe was packed with soil. The soil buildup was not removed during CIP because the cleaning velocity was insufficient or because the dead end was more than one pipe diameter.

During construction, piping should be welded using sanitary welding procedures including (for stainless steel) butt welding with an inert gas purge. Pipelines should be self-draining and without high spots or domes where air can become trapped and prevent cleaning solutions from touching those parts of the pipe.

For processing equipment the American Meat Institute created a task force to improve equipment design with the goal of eliminating harborage areas where microorganisms can collect and grow. The result is a set of guidelines, the Ten Principles of Sanitary Design (Stout, 2003). These principles, for food processing equipment, are as follows:

1. *Cleanable to microbiological level.* A piece of equipment must be more than just visually cleanable – over the entire life of the equipment.

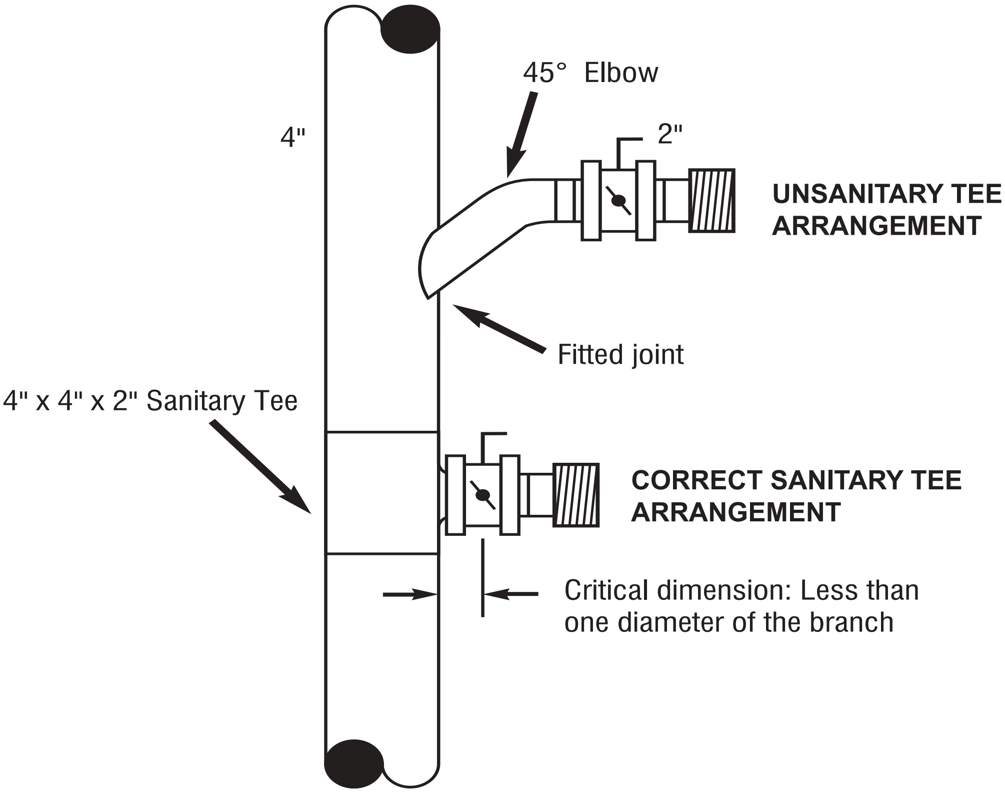

45° Elbow
2"
4"
UNSANITARY TEE ARRANGEMENT
Fitted joint
4" x 4" x 2" Sanitary Tee
CORRECT SANITARY TEE ARRANGEMENT
Critical dimension: Less than one diameter of the branch

Figure 7. Eliminating dead ends with sanitary design.

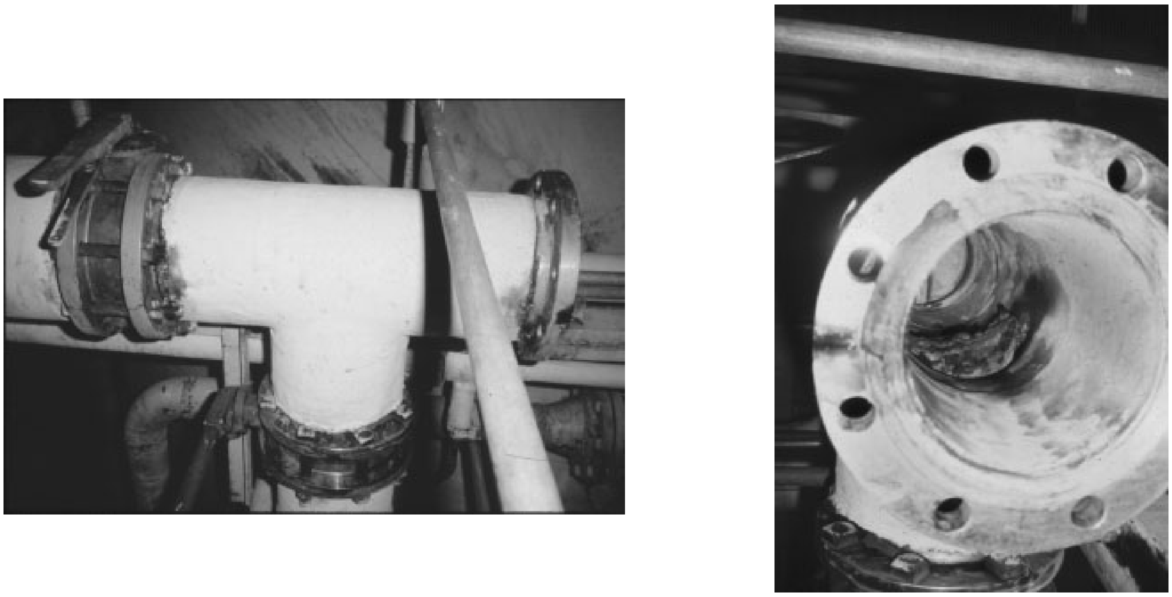


Figure 8. Buildup of soil in the dead end branch of a tee.

2. *Made of compatible materials*. Construction materials must be compatible with the product, the environment and with the cleaning materials.
3. *Accessible for inspection, maintenance and cleaning*. A person without tools must be able to inspect and clean the equipment.
4. *No product or liquid collection*. The equipment must be self draining with no areas where water or product can accumulate.
5. *Hollow areas hermetically sealed*. Hollow areas must be eliminated or hermetically sealed by continuous seam welding to eliminate any area where soil could accumulate.
6. *No niches*. There may be no harborage points: cracks, crevices, pits, dead ends, or niches. This encompasses sanitary welding in process pipelines.
7. *Sanitary operational performance*. The equipment must perform so that it does not harbor bacteria or contribute to microbiological contamination.
8. *Hygienic design of maintenance enclosures*. Control panels, junction boxes, belt guards, gear enclosures etc. are designed to ensure that neither water nor product accumulate in or on the enclosure.
9. *Hygienic compatibility with other plant systems*. Any new piece of equipment must be compatible with steam, air, water and other utilities and systems in the plant.
10. *Validate cleaning and sanitizing protocols*. The equipment manufacturer and equipment purchaser must work together during the equipment design stage to design cleanability into the machine before it is built.

THE CLEANING LOOP

A cleaning loop is the path that the cleaning solution flows during a CIP cycle. It begins at the CIP system and ends at the CIP system (Figure 9). Designing and planning cleaning loops is essential to achieving effective cleaning along the entire path that product follows through the plant.

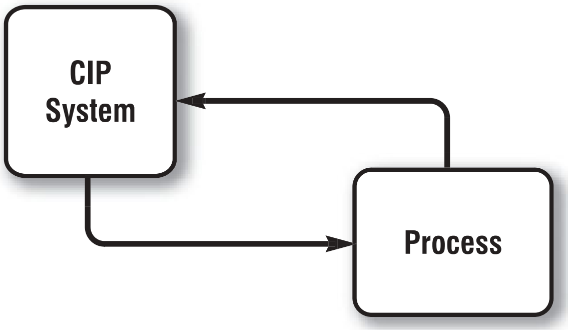


Figure 9. The cleaning loop.

Sanitary design is not just about individual tanks or pieces of equipment, it is about processing systems. Cleaning a fermentor, for example, encompasses much more than the tank itself. It includes the external heat exchanger, the product inlet and product outlet pipes, CO_2 collection lines, additive addition lines, pressure relief valves, and more. “How do we clean it?” and “what cleaning loops are necessary?” are key questions that must be asked early in the design stage.

Completely cleaning a fermentor system requires not one, but several cleaning loops (Figures 10a-10d). Some of these loops and their separate requirements include:

- Mash filling pipelines (minimum 3 m/s CIP velocity).
- Fermentor CIP sprayhead (pressure requirement).
- External heat exchanger loop (1.5 times normal process flow).
- Fermented product emptying pipeline (minimum 3 m/s CIP velocity).

The loops must be planned such that there are no lengths of pipe that lie between two loops and are therefore not included in either loop. Ideally, loops will overlap.

Validation of cleaning

When the cleaning process is complete we must know if the equipment got cleaned properly before it is committed to a new batch of product. If the final product is infected, we know that cleaning was not effective but it is then too late.

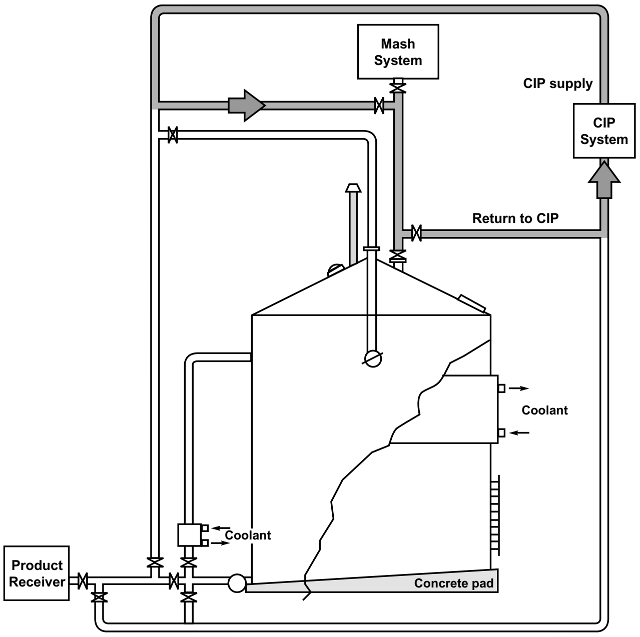

Mash System
CIP supply
CIP System
Return to CIP
Coolant
Coolant
Product Receiver
Concrete pad

Figure 10a. The mash filling pipeline loop.

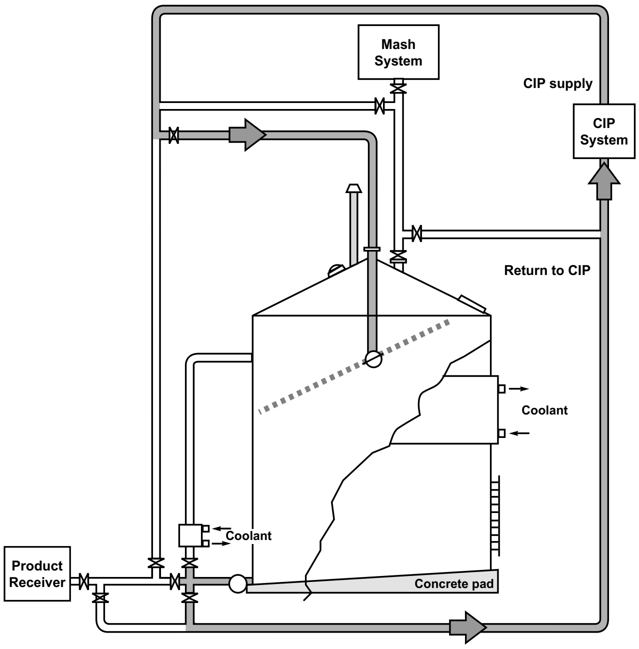

Mash System
CIP supply
CIP System
Return to CIP
Coolant
Coolant
Product Receiver
Concrete pad

Figure 10b. The fermentor CIP sprayhead loop.

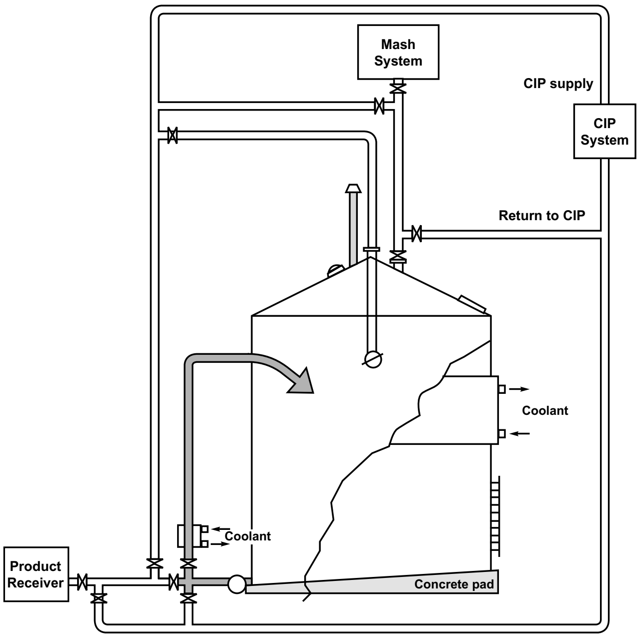

Mash System
CIP supply
CIP System
Return to CIP
Coolant
Coolant
Product Receiver
Concrete pad

Figure 10c. The external heat exchanger loop.

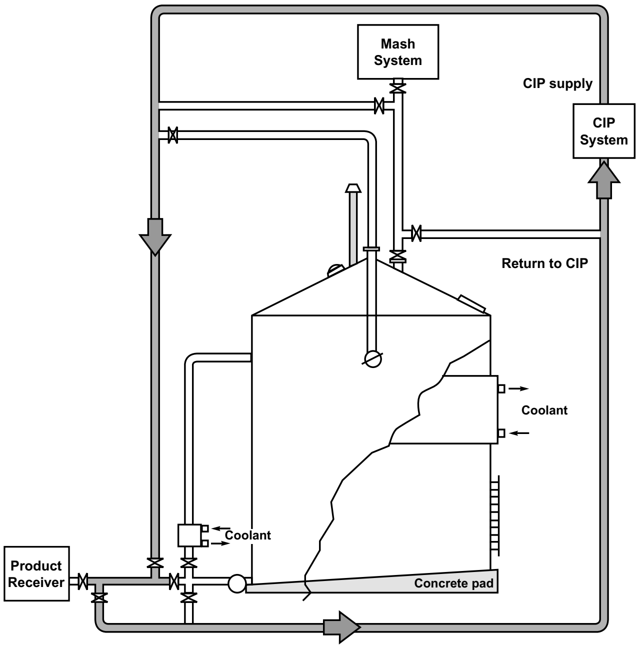

Mash System
CIP supply
CIP System
Return to CIP
Coolant
Coolant
Product Receiver
Concrete pad

Figure 10d. The fermented product loop.

Temperature, pressure and flow records can be used to document that the CIP programme ran successfully. If automation does not provide these reports, the parameters can be checked and recorded by the operators. These records are often part of a formal HACCP program.

Visual inspection may be possible from manways, inspection ports or windows. This immediately provides an indication of gross deficiencies in the cleaning process and appropriate measures can be taken before the next batch is started.

Microbiological swabs and plating, along with ATP bioluminescence swabs, are more specific indicators of successful cleaning and sanitizing. An ATP bioluminescence swab indicates the amount of soil left on a surface that may look 'clean' (Ehrenfeld *et al.*, 1996). A big advantage is that the result is known in less than one minute. Again, appropriate action (repeat the CIP process) can be taken. Microbiological swabs and plating can tell us how much of what organisms are present, but results are not known for 2-5 days. Newer rapid methods are shortening this time.

Examples of CIP cycles

A TYPICAL CIP CYCLE FOR TANKS AND PIPELINES

A. *Prerinse to the sewer.* Removes gross amounts of soil before the main wash. It is common to save the water rinse (item C) and reuse it for this step. This practice conserves water, and the rinse water usually has a small amount of detergent in it that enhances the effectiveness of the prerinse.

B. *Alkaline (caustic) wash.* For organic and oily soils, caustic soda is most commonly used. It often has additives to improve wetting and rinsing properties. Strengths from 1 to 3 % by weight are common and it is used at temperatures from ambient up to 180°F. It is a more effective cleaner at the warmer temperatures.

C. *Water rinse*. Rinses out the washing water. It is often saved for reuse as the prerinse. The source should be fresh, clean water so as not to recontaminate.

D. *Acid wash and water rinse (optional).* This additional cleaning is done to prevent scale formation, for example in beer kegs, or to remove scale deposits.

E. *Sanitizing rinse.* A sanitizing agent may be injected into the clean rinse water to reduce microorganisms on the clean surfaces to an acceptable level.

CLEANING PROCEDURE FOR CO_2 SCRUBBER

A. Stop flow of CO_2 through the scrubber and remove CO_2 from inside the scrubber by venting or rinsing with fresh water. Residual CO_2 in the scrubber will neutralize the caustic solution.

B. Make a caustic soda solution 2 to 3% by weight at ambient temperature or preferably warmed to 100 to 120°F. Make enough to maintain a level in the bottom of the scrubber when recirculating.

C. Circulate the caustic solution through the scrubber sprayer for at least 1 hr. Several hours may be required depending on the soil load.

D. After 15 to 30 minutes take a sample of the caustic and titrate to make sure it has not been neutralized by residual CO_2 in the scrubber. If less than 2% strength, add more caustic to get 2 to 3%.

E. Drain caustic, then rinse using burst rinse (water for 30 sec, drain for 3 min.). Repeat burst rinse cycle until rinse water shows no caustic when tested with phenylphthalein or litmus paper.

F. Prepare acid wash, 1 to 2%, using Scalebite™ at ambient temperature. Circulate for 1 hr.

G. Drain acid solution and rinse using burst rinse, three cycles.

H. Prepare Iotech™ solution, 25 ppm at ambient temperature, and recirculate for 30 mins.

I. Drain Iotech™ solution and rinse using burst rinse, three cycles.

J. Scrubber is ready to bring back on stream.

PROCEDURES FOR DE-SCALING FERMENTORS

The following is an approach to descaling tanks and equipment after beerstone or scale has accumulated over a period of time. Since scale amounts and compositions vary from time to time and place to place, some trial and error is usually required.

A. *Prerinse the tank to remove heavy soil.* Use fresh water or water with a little caustic, like the saved final rinse water from washing another tank. Do not save this liquid in the CIP system.

B. *Main caustic wash.* Caustic strength at 3 to 5%, temperature 120 to 190°F. This will be similar to the normal caustic wash and when finished, the tank should be clean except for the scale or beerstone. Addition of sodium hypochlorite (bleach) to caustic solutions may help to attack the scale. It is used at concentrations of 200 to 400 ppm as active chlorine. CAUTION – never mix hypochlorite (bleach) with acid cleaners.

C. *Scale-removing caustic wash.* Use caustic as in step 2 but with addition of Scaleban™ as a supplement. For infrequent de-scaling, add 5 oz Scaleban™ for every gallon of the caustic solution. Same temperatures, 120 to 190°F. Warmer is usually better. Circulate several hours if possible. Use kit to titrate EDTA, the active ingredient in Scaleban™. Add more Scaleban™ if EDTA gets depleted. For routine scale control, 1 to 3 oz of Scaleban™ is added to each gallon of the normal caustic solution.

D. *Rinse with clean water. Inspect visually.* Note: when tank is wet you may not see the scale. When it dries, the scale is more visible.

E. *Scale-removing acid wash.* If scale remains after the Scaleban™ wash, an acid cleaner may be required. Scalebite™ is a formulated acid cleaner that is used between 1 and 3% in water. It is recirculated several hours. Note: Scalebite™ is safe with stainless steel but does attack mild steel, especially at warmer temperatures. Sulfamic acid is also effective against some scales. Like Scalebite™, it is used in concentrations between 1 and 3 %, temperatures up to 140°F.

Problem areas

When a contamination problem develops, one of the first questions asked is 'How did the infection develop?' One of the most difficult challenges in industrial sanitation is in keeping a continuous fermentation system free of infection. Doug Banks from New Zealand's Dominion Breweries, which has successfully operated breweries with continuous fermentation of beer and no infection for many years, classifies sources of contamination into two groups, point sources and dead ends, based on years of experience:

Point sources are sources that occur only from time to time. They may appear and then disappear pretty much at random. Some point sources include:

- Bad pump seals that allow contamination to be drawn in from the outside environment by a Venturi effect of the flowing liquid.
- A piece of equipment that was missed during regularly scheduled cleaning.
- An employee with dirty boots, *etc*. brings contamination into the system by climbing into a clean tank or some other area to make some final adjustment or repair.
- A cleaning sprayball has become plugged.
- Sample ports have been forgotten during cleaning and have become infected.
- A solution used for sanitizing small pieces of equipment out of place has stood for days, losing its effectiveness.
- The cleaning solution used was too dilute.
- The wind blows contaminated air or an aerosol into equipment when it is temporarily opened after cleaning and before use.
- Insects carry contamination into a piece of equipment temporarily left open.

Dead ends are sources of contamination that are always in contact with the process stream. Usually these are the most serious causes of contamination. Some examples are:

- Dead ends in piping that cannot be thoroughly cleaned.
- Scale built up inside fermenting tanks or coolers. Scale cannot be cleaned because the cleaning compounds cannot penetrate the scale once it is thick enough.
- Carbon dioxide vent lines on fermentors into which fermenting liquid has flowed or been sprayed as an aerosol.
- Faulty check valves
- Contaminated yeast
- Poor drainage of cleaning solution from a tank while it is being cleaned.
- Seats on valves that have worn and begun to develop cracks
- Non-sanitary pipe connections, especially pipe threads. These will trap liquid in the threads that cannot be removed by cleaning.

Some of the likely ultimate sources of contamination include:

- Grains being brought into a plant carry large numbers of a variety of microorganisms
- People and the food they bring into the plant
- Insects

The most likely sources of contamination, though, are within the plant itself. Infection sources are often found in scale, dead ends, even in coolers, spilled material left on the ground, material in sewers that can be splashed into the air as an aerosol, dirty and contaminated carbon dioxide lines and aerosols created when opening contaminated equipment such as perhaps the beer well.

Hidden issues related to chemistry occur when cleaning tanks that have a CO_2 atmosphere. The CO_2 will react with caustic to form sodium carbonate, a cleaner that is much weaker than caustic soda (Figure 11). However, if this reacted solution is analyzed by the normal single end-point titration, the effect is not detected. Carbonate titrates just like the hydroxide. This problem can be overcome by replenishing the reacted caustic or by venting the vessel completely before cleaning. The correct titration uses barium chloride first, then acid. The method can be found in Alltech's Laboratory Manual (Alltech, 2001).

$$2\,NaOH + CO_2 \longrightarrow Na_2CO_3 + H_2O$$

Sodium hydroxide (an excellent cleaner)

Sodium carbonate (a fairly good cleaner)

Figure 11. Dilution of caustic by carbon dioxide.

A CASE STUDY: CLEANING A FERMENTOR

Cleaning the inside of a fermentor with a sprayhead and cleaning the tank outlet (Figure 12)

The normal flow through a sprayhead is around 100 gallons/min (about 380 liters/min). For a 6-inch fermentor outlet pipe, the minimum velocity for cleaning is not reached until the flow rate is 410 gallons/min. This is a serious cleaning deficiency and it is common to see gross accumulations of soil on the top half of these outlet pipes because they do not even fill with cleaning solution when the sprayhead is being used.

The cleaning and sanitizing program

A routine and effective microbiological sampling program should be in operation. Sampling should be planned so as to provide a complete, overall picture of the state of sanitation in the plant at any time. Special attention should be paid to an evaluation of the cleaning procedures in place. Infections usually develop in areas where cleaning is not effective or is missed. Swabs should be taken from cleaned equipment. An ATP detecting luminometer can provide more immediate evaluation on the effectiveness of cleaning. Strengths of cleaning and sanitizing solutions should be measured routinely, as well as temperatures of cleaning solutions.

Sanitation Quality Control is like preventive maintenance for the product. It is troubleshooting designed to identify potential problems so that they can be corrected before damage is done to the product. There are four basic components in an effective sanitation quality control program:

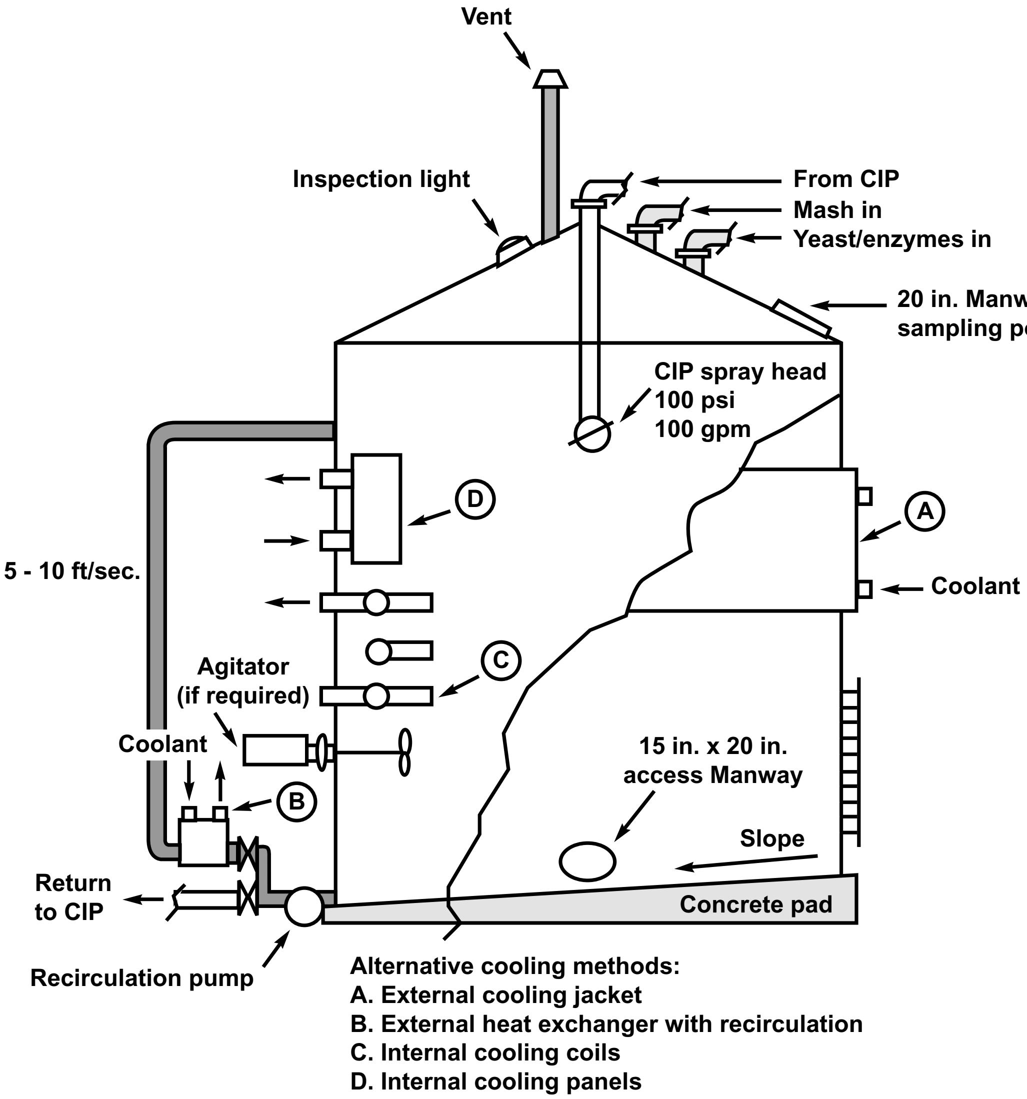

Vent
Inspection light
From CIP
Mash in
Yeast/enzymes in
20 in. Manway/ sampling port
CIP spray head 100 psi 100 gpm
D
A
Coolant
5 - 10 ft/sec.
C
Agitator (if required)
Coolant
B
15 in. x 20 in. access Manway
Slope
Return to CIP
Concrete pad
Recirculation pump
Alternative cooling methods:
A. External cooling jacket
B. External heat exchanger with recirculation
C. Internal cooling coils
D. Internal cooling panels

Figure 12. Cleaning a fermentor.

A. The Sanitation Plan

1. Master sanitation schedule
2. Times, temperatures, materials, strengths for every cleaning job
3. Detailed written instructions for every cleaning job

B. Measurements and Standards

Every cleaning and sanitizing process should have, as an objective, the achievement of an end result that can be measured and compared to a predetermined standard.

1. *Visual inspection* - gives a 'go/no go' result immediately. Wet surfaces can appear clean when they are actually dirty, so the visual inspection is best done when the surface is dry. This should also include 'white glove' checks.
2. *ATP-detecting luminometer* - immediate quantitative results for presence of ATP, which indicates cleaning was not complete.
3. *Microbiological* - a time of 24 hrs to 1 week is required before results are known. Tests include forcing tests, swabs, pour plates and millipore filtering. Different tests are

specified at different stages of the process and each test has standard results or control limit.

C. Records

These should include: when was the cleaning really done (*vs* schedule), automatic or manual recording of temperatures and strengths, when was maintenance done (UV lights renewed, eg), problems encountered when the job was done.

D. Results

Immediate reporting of microbiological results and charting trends for statistical quality control are of key importance.

Summary

For every cleaning and sanitizing problem there is a solution, in fact there can be many solutions. Unfortunately the solutions are not available as defined recipes in a cookbook. The Four Ts, our cleaning and sanitizing bag of tricks, experience and trial and error are the tools at our disposal. We know that the program is to first remove the dirt, and then sanitize. Sanitizing dirt may work for a short time but it is not a permanent solution. We must also determine the acceptable levels of cleanliness and sanitation and then avoid excesses that are hard on the equipment and costly in terms of time, money and utilities.

We must use our knowledge of the Four Ts of cleaning and sanitizing to find a combination of time, temperature, turbulence and titration that is both effective and economical. Finally, we must realize that while CIP is the ideal, COP combined with CIP is usually the reality.

> *“Regardless of technology, the most important food plant sanitation approach is getting back to basics, which means having motivated, passionate people who know both the importance of and how to clean effectively. It comes down to training and motivation; simply put, how to use mechanical action, with the right detergent, at the right concentration for the right time at the right temperature with the right attitude, and giving people credit for the good work they do.”*
>
> (Stout, 2002).

Cleaning and sanitation is an area where the quality control principle of ‘continuous improvement’ can be effectively applied. No plant is perfectly clean. There is always room for improvement, and small improvements can accumulate into major improvements in the overall efficiency and profitability of the plant.

References

Alltech Inc. 2001. Laboratory Methods in Brewing and Distilling. Alltech Technical Publications, pg 3-16.

Anon. 1992. 3-A Accepted Practices For Permanently Installed Product And Solution Pipelines And Cleaning Systems Used In Milk and Milk Product Processing Plants, Number 605-04. Dairy, Food and Environmental Sanitation, Volume 12, No. 2.

Czechowski, M.H. and M. Banner. 1992. Control of biofilms in breweries through cleaning and sanitizing.Tech. Q. Master Brewers Assoc. of Am. 29(3):86-88.

Ehrenfeld, E.E., J. Scheld, S.A. Miller and C.R. Carpenter. 1996. Use of ATP-bioluminescence in the brewing industry. Tech. Q. Master Brewers Assoc. Am. 33(1):59-62.

Kretsch, J. 1994. Practical considerations for brewery sanitation. Tech. Q. Master Brewers Assoc. Am. 31(4):124-128.

McCabe, J.T. 1999. The Practical Brewer, 3rd Edition. Master Brewers Association of the Americas.

Peter, W.A. 1983. Designing for automation. MBAA Technical Quarterly 20(1):22.

Stout, J. 2002. Perspective on practices in food plant sanitation and hygiene. Food Safety Magazine, April/May pg.20.

Stout, J. 2003. 10 Principles of equipment design for ready to eat food processing operations. Food Safety Magazine, June/July, pg. 18.

Recovery

Chapter 22

Ethanol distillation: the fundamentals

P.W. MADSON

KATZEN International, Inc., Cincinnati, Ohio, USA

Fundamentals of a distilling system

Certain fundamental principles are common to all distilling systems. Modern distillation systems are multi-stage, continuous, countercurrent, vapor-liquid contacting systems that operate within the physical laws that state that different materials boil at different temperatures.

Represented in Figure 1 is a typical distillation tower that could be employed to separate an ideal mixture. Such a system would contain the following elements:

a. a feed composed of the two components to be separated,

b. a source of energy to drive the process (in most cases, this energy source is steam, either directly entering the base of the tower or transferring its energy to the tower contents through an indirect heat exchanger called a reboiler),

c. an overhead, purified product consisting primarily of the feed component with the lower boiling point,

d. a bottoms product containing the component of the feed possessing the higher boiling point,

e. an overhead heat exchanger (condenser), normally water-cooled, to condense the vapor resulting from the boiling created by the energy input. The overhead vapor, after condensation, is split into two streams. One stream is the overhead product; the other is the reflux which is returned to the top of the tower to supply the liquid downflow required in the upper portion of the tower.

The portion of the tower above the feed entry point is defined as the 'rectifying section' of the tower. The part of the tower below the feed entry point is referred to as the 'stripping section' of the tower.

The system shown in Figure 1 is typical for the separation of a two component feed consisting of ideal, or nearly ideal, components into a relatively pure overhead product containing the lower boiling component and a bottoms product containing primarily the higher boiling component of the original feed.

If energy was cheap and the ethanol-water system was ideal, then this rather simple distillation system would suffice for the separation of the beer feed into a relatively pure ethanol overhead product and a bottoms product of stillage, cleanly stripped of its ethanol content. Unfortunately, the ethanol-water (beer) mixture is not an ideal system. The balance of this chapter

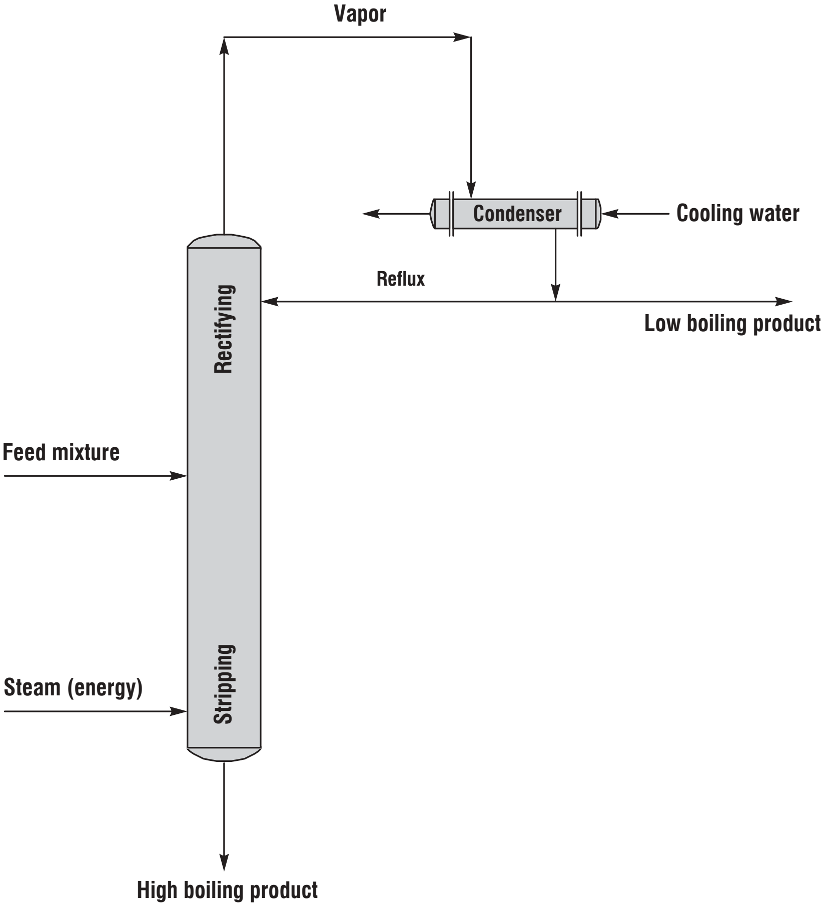


Figure 1. Ideal distillation system.

will be devoted to a description of the modifications required of the simple distillation system in order to make it effective for the separation of a very pure ethanol product, essentially free of its water content.

Figure 2 expands on Figure 1 by showing some additional features of a distillation tower. These are:

a. The highest temperature in the tower will occur at the base.

b. The temperature in the tower will regularly and progressively decrease from the bottom to the top of the tower.

c. The tower will have a number of similar, individual, internal components referred to as ‘trays’ (these may also be described as stages or contactors).

d. Vapor will rise up the tower and liquid will flow down the tower. The purpose of the tower internals (trays) is to allow intimate contact between rising vapors and descending liquids correlated with separation of vapor and liquid.

Figure 3 shows a vapor-liquid equilibrium diagram for the ethanol-water system at atmospheric pressure. The diagram shows mole percent ethanol in the liquid (X axis) *vs* mole percent ethanol in the vapor (Y axis). The plot could also be made for volume percent in the liquid *vs* volume percent in the vapor and the equilibrium curve would only be slightly displaced from that shown in Figure 3. Mole percent is generally used by engineers to analyze vapor/liquid separation systems because it

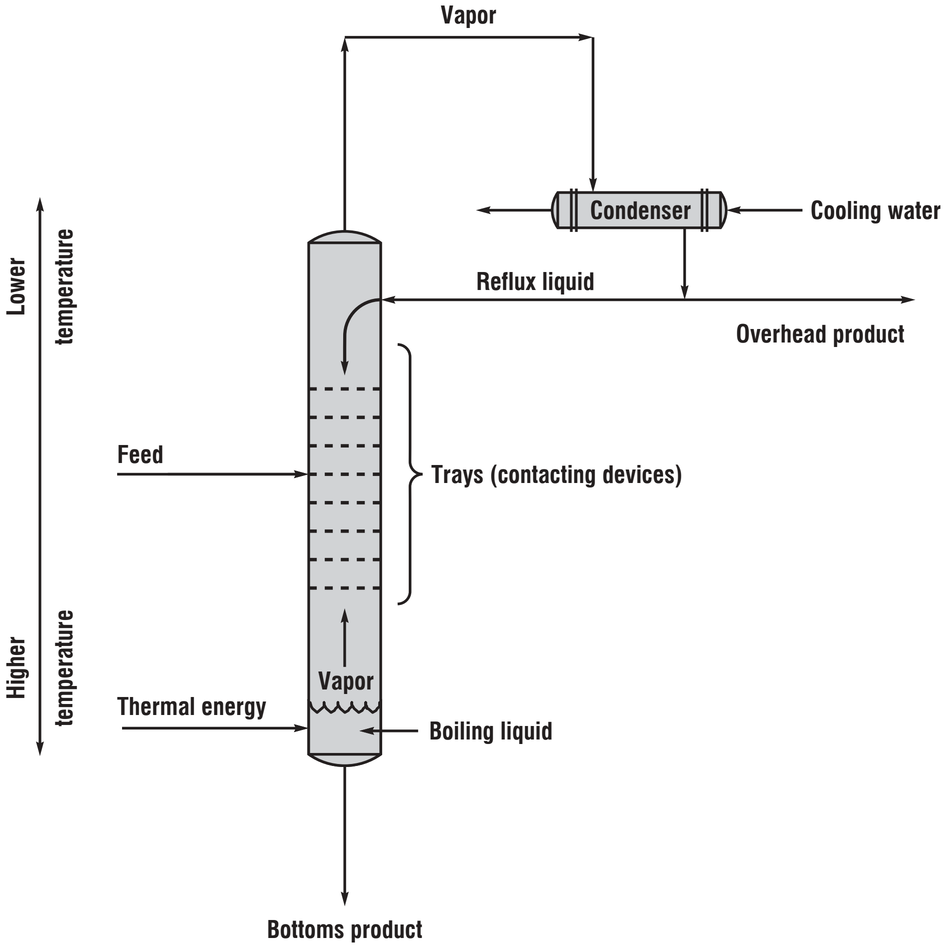


Figure 2. Typical distillation relationships.

relates directly to molecular interactions, which more closely describe the process occurring in a distillation system.

Analysis of the ethanol-water distillation system is mathematically straightforward when using molar quantities rather than the more common measurements of volume or weight. This is because of an energy balance principle called 'constant molal overflow'. Essentially, this principle states that the heat (energy) required to vaporize or condense a mole of ethanol is approximately equal to the heat (energy) required to vaporize or condense a mole of water; and is approximately equal to the heat (energy) required to vaporize or condense any mixture of the two. This relationship allows the tower to be analyzed by graphic techniques using straight lines. If constant molal overflow did not occur, then the tower analysis would become quite complex and would not lend itself easily to graphic analysis.

Referring to Figure 3, a 45° line is drawn from the compositions of 0, 0 to 100% and 100%. This 45° line is useful for determining ranges of compositions that can be separated by distillation. Since the 45° line represents the potential points at which the concentration in the vapor equals the concentration in the liquid, it indicates those conditions under which distillation is impossible for performing the separation. If the equilibrium curve contacts the 45° line, an infinitely large distillation tower would be required to distill to that composition of vapor and liquid. Further, if the equilibrium curve crosses the 45° line, the mixture has formed an azeotrope. This means that even if the tower were infinitely large with an infinite amount of energy, it would be impossible to distill past that point by simple rectification.

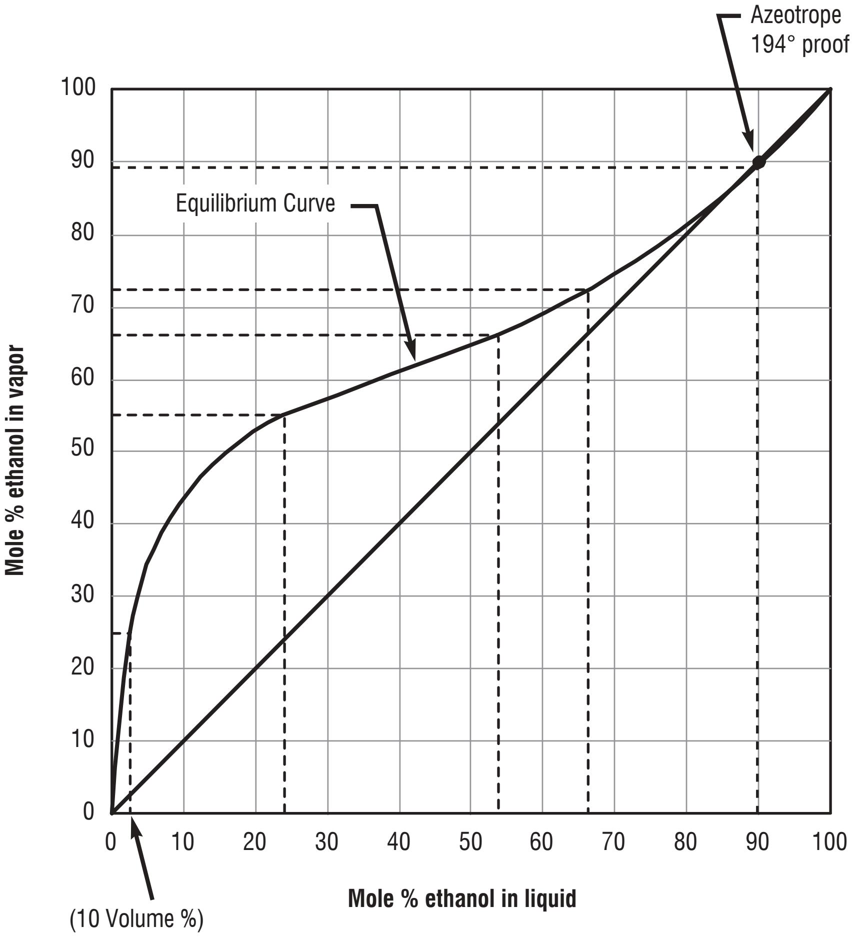


Figure 3. Vapor/liquid equilibrium for the ethanol/water system at atmospheric pressure.

Consider a very simple system consisting of a pot filled with a mixture of ethanol in water (a beer) containing 10% by volume ethanol (3.3 mole %). This composition is identified in the lower left portion of Figure 3. A fire could be kindled under the pot, which would add thermal energy to the system. The pot would begin to boil and generate some vapor. If we gathered a small portion of the vapor initially generated and measured its ethanol content; we would find about 24 mole % ethanol (53 volume %). If we condense this vapor (note: there will be only a small amount of this vapor), boil it in a second pot and again collect a small amount of the initial vapor generated, this second vapor would contain about 55 mole % (83 volume %) of ethanol (see Figure 3). If we should continue this simplified process to a third and fourth collection of small amounts of vapor, analysis would reveal that each successive portion of vapor would become richer in ethanol.

Thus we have created a series of steps by which we keep increasing the ethanol content of the analyzed sample, both liquid and vapor. Unfortunately, this oversimplified process is idealized; and practically speaking, is impossible. However if we had supplied our original pot with a continuous supply of ethanol-water feed and vapor generated in the first pot was continuously condensed and supplied to the second pot, etc. then the process becomes similar to the industrial distillation tower operation shown in Figure 2.

How far can this process be extended? Could we produce pure ethanol by continuously extending our process of boiling and reboiling?

The answer is, no! We would finally reach a point in one of the downstream pots, where the vapor boiling from the liquid was of the same composition as the liquid from which it was being generated. This unfortunate consequence limits our ability to produce anydrous ethanol from a dilute ethanol-water feed. What we finally encounter in our simplified process is the formation of an *azeotrope*. This is a concentrated solution of ethanol and water that when boiled produces a vapor with a composition identical to the composition of the liquid solution from which it originated.

In summary then, we are limited in ethanol-water purification in any single multistage distillation tower to the production of azeotropic ethanol-water mixtures. These azeotropic solutions of ethanol and water are also known as constant boiling mixures (CBM) since the azeotropic liquid will have the same temperature as the azeotropic equilibrium vapor being boiled from itself. Without some sort of drastic process intervention, further ethanol purification becomes impossible. The question then becomes: What can we do to make it possible to produce anhydrous ethanol? Methods of doing so will be covered later in this chapter.

Figure 4 depicts the structure of the distillation process by dividing the vapor/liquid equilibrium information into three distinct zones of process and equipment requirements: stripping, rectifying and dehydration. This division is the basis for the design of equipment and systems to perform the distillation tasks.

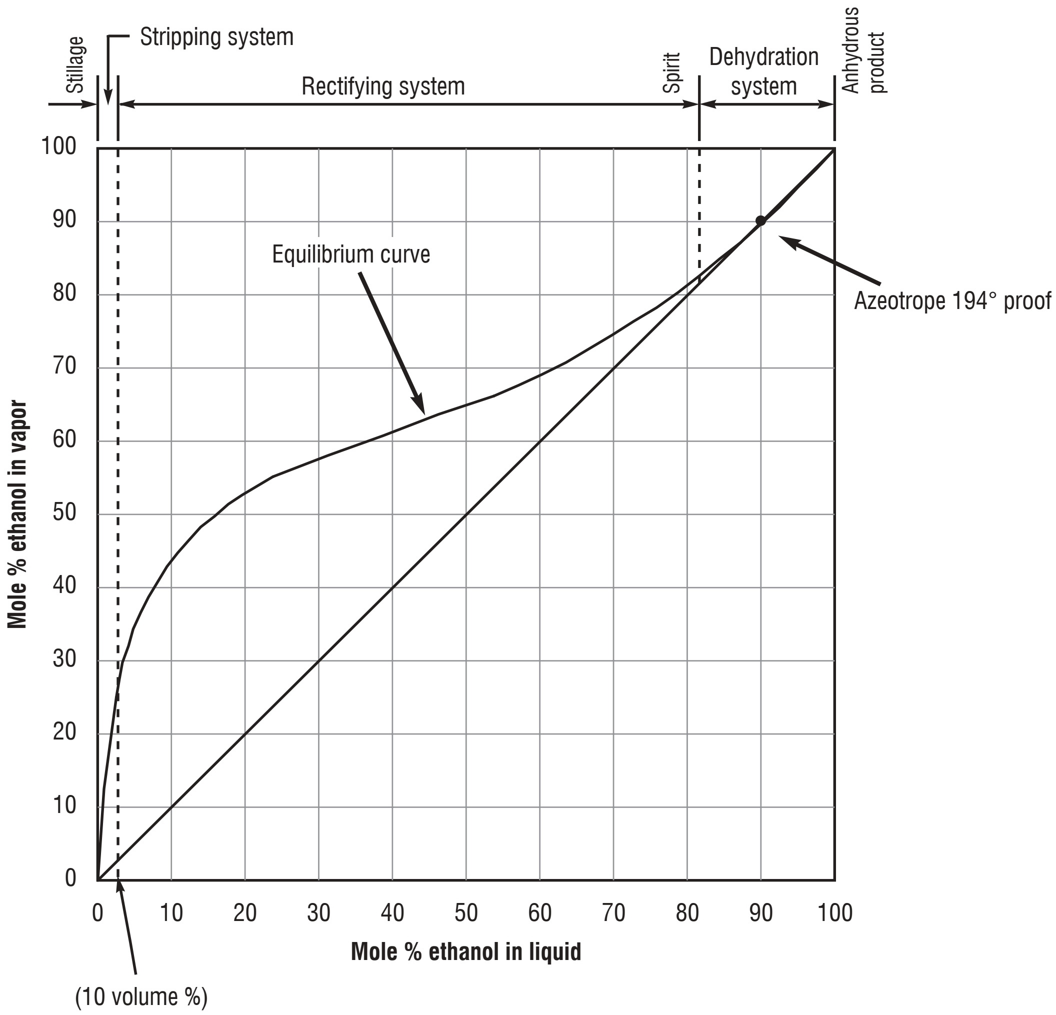


Figure 4. Structuring the distillation system strategy.

Considerations in preliminary design

The engineer, given the assignment of designing a distillation tower, is faced with a number of fundamental considerations. These include:

a. What sort of contacting devices should be employed? (e.g. trays or packing). If trays are chosen, what type will give the most intimate contact of vapor and liquid?

b. How much vapor is needed? How much liquid reflux is required? (What ratio of liquid: vapor is required?)

c. How much steam (energy) will be required?

d. What are the general dimensions of the distillation tower?

DISTILLATION CONTACTORS

Trays are the most common contactor in use. What are the functions expected of tray contactors in the tower? Figure 5 depicts a single tray contactor in a distillation tower and shows the primary functions desired:

- mixing rising vapor with a falling fluid
- allow for separation after mixing
- provide path for liquid to proceed down the tower
- provide path for vapor to proceed up the tower

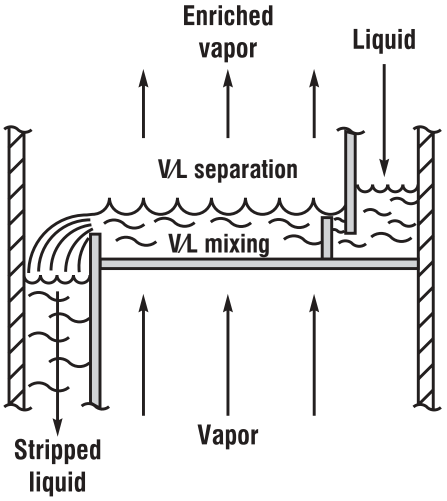

Enriched vapor
Liquid
V/L separation
V/L mixing
Stripped liquid
Vapor

Figure 5. Distillation tray functions.

Figure 6 depicts a perforated tray contactor with certain accoutrements required to control the flow of liquid and vapor and to assure their intimate contact. Another type of tray contacting device, the disc-and-donut or baffle tray is shown in Figure 7. The characteristics of this type of contactor make it especially useful for distilling materials such as dry-milled grain beer, which would foul ordinary trays such as perforated, valve, etc.

ENERGY ANALYSIS

In addition to the selection of the basic contacting device, the energy requirement must be

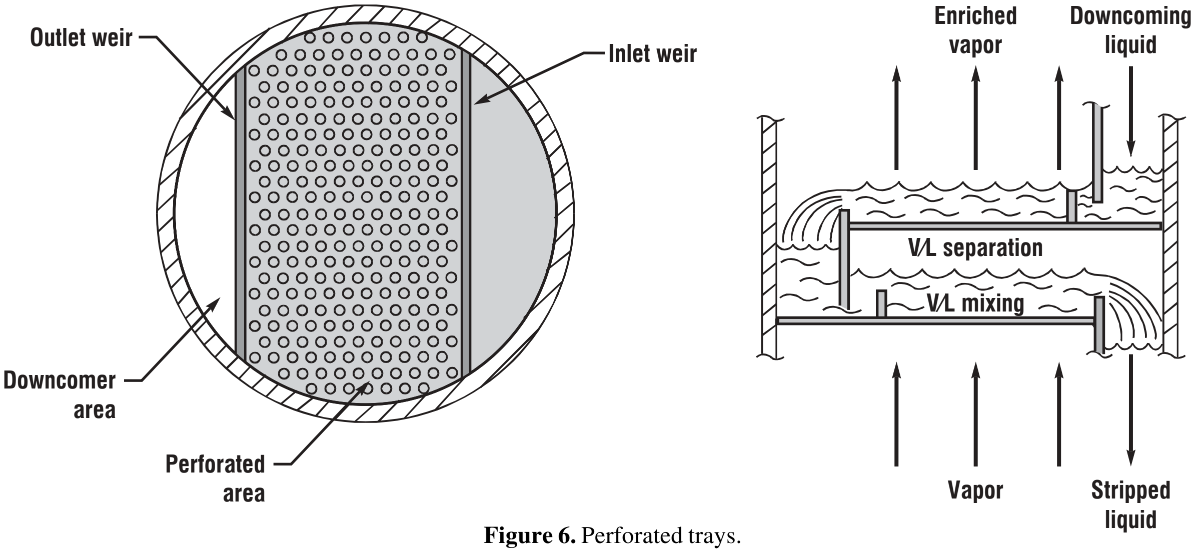

Outlet weir
Inlet weir
Downcomer area
Perforated area
Enriched vapor
Downcoming liquid
V/L separation
V/L mixing
Vapor
Stripped liquid

Figure 6. Perforated trays.

established. This is accomplished by analyzing the vapor/liquid equilibrium data from Figure 4, for the liquid:vapor flow ratio to perform a continuous series of steps within the limits of the equilibrium curve. Table 1 demonstrates a simplified procedure to calculate the approximate energy requirement from the liquid:vapor flow ratio that will be employed in the tower design. Repetition of this type of calculation for different conditions produces a

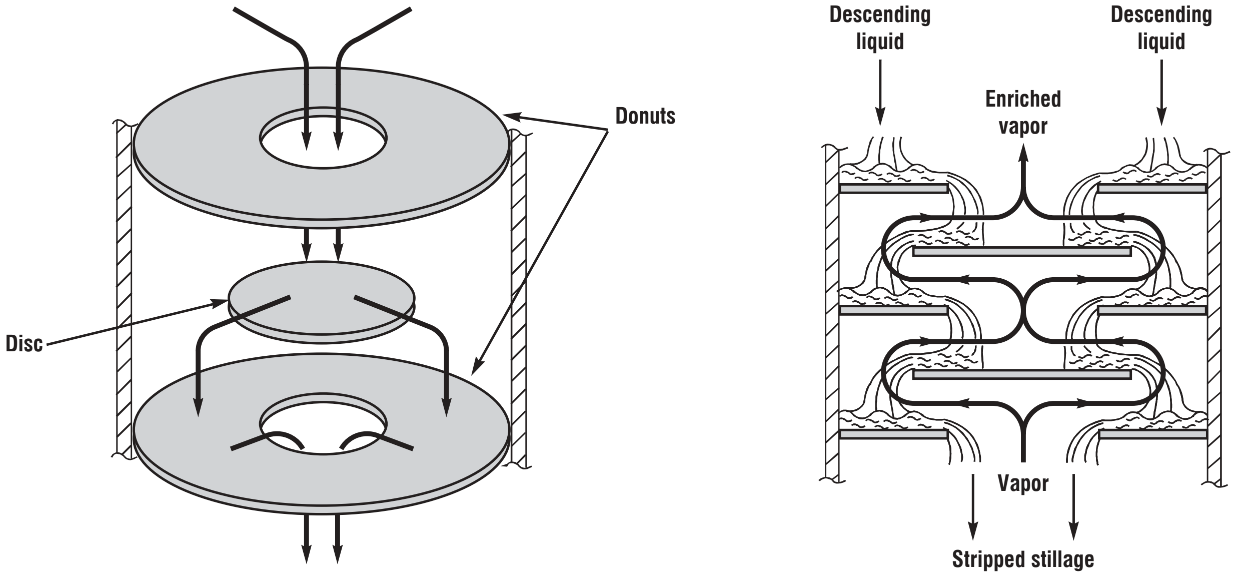


Figure 7. Disc-and-donut trays.

Table 1. Simplified calculations for steam requirements for ethanol distillation.

Example 1. Calculate the steam required (lbs/gallon of product) to strip 100 gpm of a 10% volume beer (90 gpm water/ 10 gpm ethanol).

$L/V^* = 5.0$ (typical for a 10% volume beer) or $L = 5\ V$

$$L = 90 \text{ gpm} \bullet \frac{500 \text{ lbs/hr}}{\text{gpm}} = 45{,}000 \text{ lbs/hr} = 5 \bullet V$$

Therefore, $V = 9{,}000$ lbs/hr (steam)

$$\text{And: } \frac{9{,}000 \text{ lbs/hr (steam)}}{10 \text{ gpm (product)}} \bullet \frac{\text{hr}}{60 \text{ min}} = 15 \text{ lbs steam/gallon of product}$$

Example 2. Calculate the steam required to strip 100 gpm of a 5% volume beer (95 gpm water/5 gpm ethanol).

$L/V = 6.33$ (typical for a 5% volume beer) or $L = 6.33 \bullet V$

$$L = 95 \text{ gpm} \bullet \frac{500 \text{ lbs/hr}}{\text{gpm}} = 47{,}500 \text{ lbs/hr} = 6.33 \bullet V$$

Therefore, $V = 7{,}500$ lbs/hr (steam)

$$\text{And: } \frac{7{,}500 \text{ lbs/hr (steam)}}{5 \text{ gpm (product)}} \bullet \frac{\text{hr}}{60 \text{ min}} = 25 \text{ lbs steam/gallon of product}$$

---

*L and V are liquid and vapor flow rates, respectively, expressed in lb-mole per hr.

Note: at base of column use simplifying assumption of water (L) and steam (V). Therefore: $\frac{L \text{ (lb-mole/hr)}}{V \text{(lb-mole/hr)}} = \frac{L \text{ (lbs/hr)}}{V \text{(lbs/hr)}}$

design chart like that shown in Figure 8 for the ethanol-water system. Such a graph is useful when calculations are needed to ascertain technical and economic feasibility and preliminary conditions for the design.

Figure 9 demonstrates how the liquid:vapor flow ratio, in connection with the number of stages (theoretically ideal trays) required for a specified separation between ethanol and water, is graphically determined. Note that the stages are constructed by drawing straight lines vertically and horizontally between the equilibrium curve (previously determined experimentally) and the operating lines. For an ethanol stripper/rectifier, there are two operating lines: one for the rectification section and one for the stripping section. The operating lines represent the locus of concentrations within the distillation tower of the passing liquid and vapor streams. The operating lines for a given tower are based on the energy input, as calculated and represented in Figure 8. Because of the principle of constant molal overflow, the operating lines can be represented as straight lines. If constant molal overflow was not valid for the ethanol/water distillation, then these lines would be curved to represent the changing ratio of liquid flow to vapor flow (in molar quantities) throughout the tower. The slope of the operating line (the ratio of liquid flow to vapor flow) is also called the *internal reflux ratio*. If the energy input to a tower is increased while the beer flow remains constant, the operating lines will move toward the 45° line, thus requiring fewer stages to conduct the distillation. Likewise if the energy input is reduced (lowering the internal reflux ratio), the operating lines will move toward the equilibrium curve, reducing the degree of separation achievable in each stage and therefore

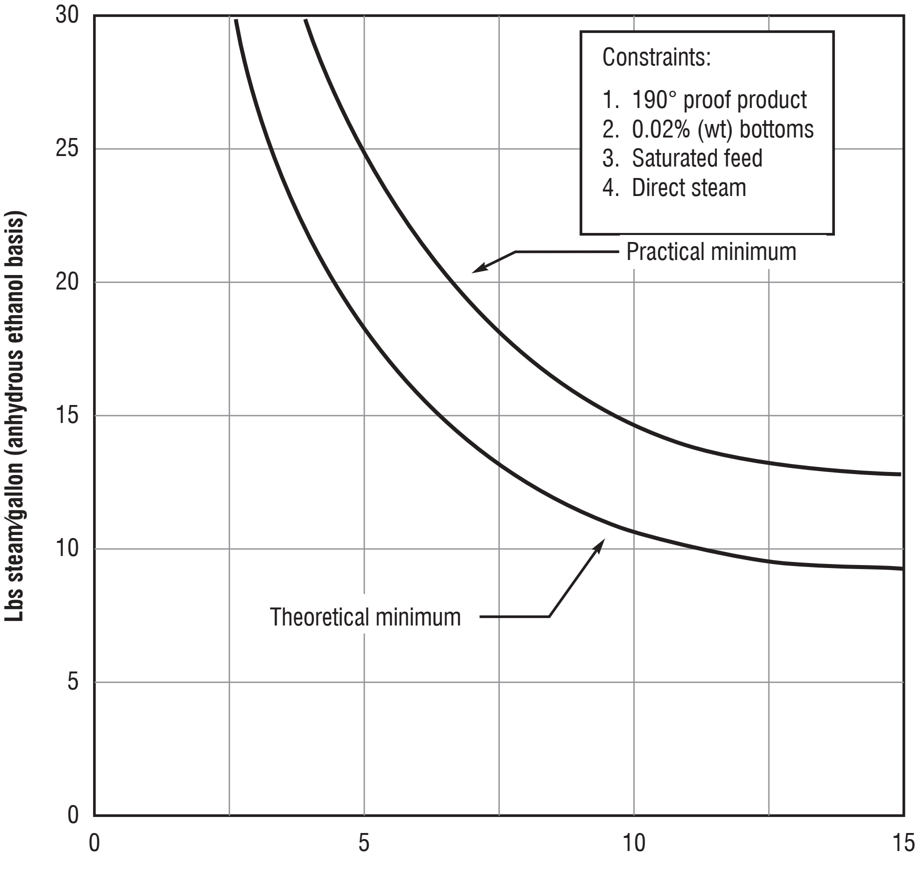


Figure 8. Steam requirements ethanol stripper/rectifier.

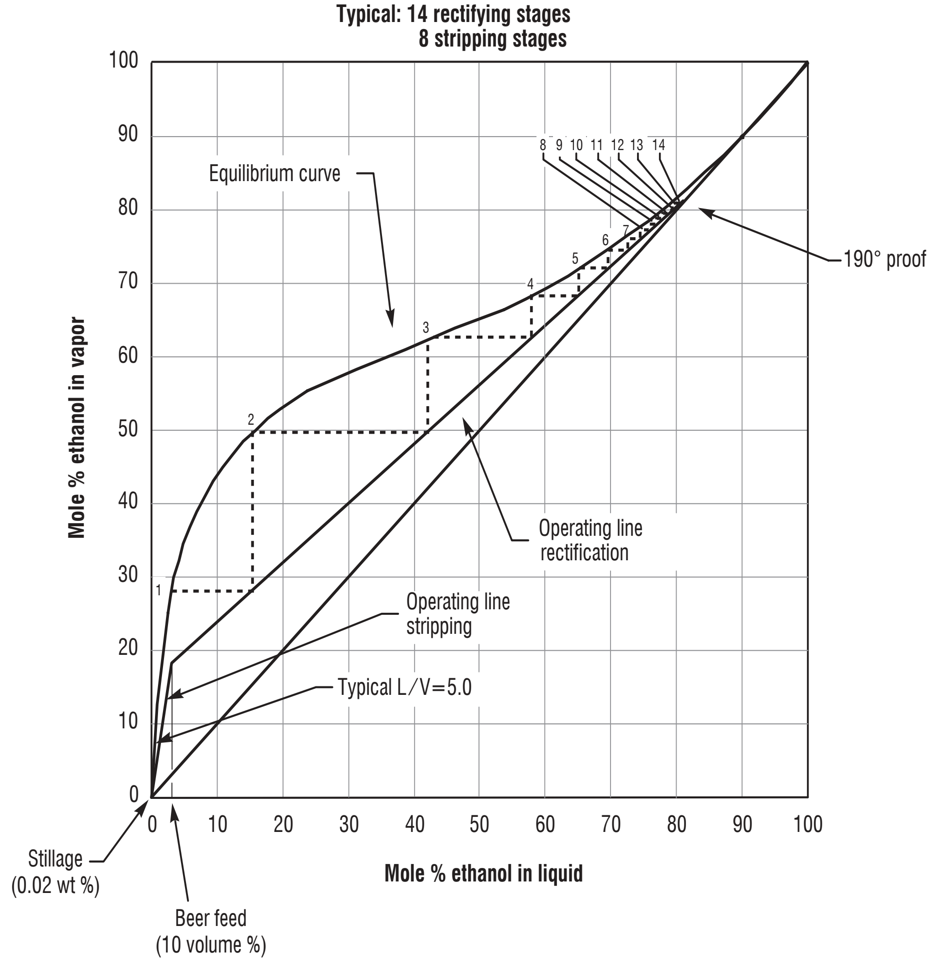


Figure 9. Vapor/liquid equilibrium stage analysis.

requiring more stages to conduct the distillation. The calculations underlying the preparation of Figure 9 go beyond the scope and intent of this text, but have been included for continuity. The dashed lines represent the graphical solution to the design calculations for the number of theoretical stages required to accomplish a desired degree of separation of the feed components. Figure 9 is referred to as a *McCabe-Thiele diagram*. For further pursuit of this subject, refer to the classical distillation textbook by Robinson and Gilliland (1950).

TOWER SIZING

The goal of the design effort is to establish the size of the distillation tower required. Table 2 shows the basic procedure to determine the diameter required for the given distillation tower. Since all of the distillation 'work' is done by the trays, the tower is actually the 'container' to surround the vapor and liquid activity that is 'managed' by the trays. Tower diameter design is, therefore, actually the design of the necessary tray diameter for proper vapor/liquid interaction and movement.

The *f* factor (vapor loading) is an empirically determined factor that depends primarily upon tray type and spacing, fluid physical properties, froth stability and surface tension at the operating conditions of the system. The proper values for *f* are determined by field observations. In summary then, the *f* factor can be described as an adjusted velocity term (units are ft/sec) that when multiplied by the square root of the density ratio of liquid to vapor, will give the allowable vapor velocity in the empty tower shell, such

Table 2. Calculations for tower sizing (base of stripper).

Example: Calculate tower diameter required for a 10% volume beer at 100 gpm (15 lbs steam/gallon of product)

W	Vapor flow rate	= 9,000 lbs/hr (steam)
P	Operating pressure at base	= 1.34 ATM
M_{AVG}	Average MW of vapor	= 18 lbs/lb-mole
ρ_L	Liquid mixture density	= 59.5 lbs/ft^3 (227 °F)
T	Absolute operating temperature	= 687 °R
D	Tower inside diameter in inches	
f	Vapor loading factor	= 0.05-0.3

Sizing equation:

$$D = 0.2085 \bullet \sqrt{\frac{W}{f \bullet \sqrt{\frac{P \bullet M_{AVG} \bullet \psi_L}{T}}}} = 0.2085 \bullet \sqrt{\frac{9000}{f \bullet \sqrt{\frac{1.34 \bullet 18 \bullet 59.5}{687}}}} = \frac{16.45}{\sqrt{f}}$$

Assuming $f = 0.16$ (specific to tray design and spacing), the tower diameter is:

$$D = \frac{16.45}{\sqrt{0.16}} = 41.125 \text{ inches}$$

Tower diameter calculation utilizes the following equation. Values for the terms are indicated above.

$$D = 0.2085 \bullet \sqrt{\frac{W}{f \bullet \sqrt{\frac{P \bullet M_{AVG} \bullet \psi_L}{T}}}}$$

The final design equation can be derived beginning with the fundamental equation:

$$u = f \bullet \sqrt{\frac{\rho_L - \rho_V}{\rho_V}}$$

Where u = average vapor velocity in empty tower shell (ft/sec)
ρ_L = liquid density (lbs/ft^3)
ρ_V = vapor density (lbs/ft^3)
f = tower vapor loading factor (ft/sec)

(Note: For most cases ρ_L is much greater than ρ_V, so that $\rho_L - \rho_V \cong \rho_L$. For example, water (steam) at 212°F and atmospheric pressure: $\rho_L = 59.8$ lbs/ft^3 and $\rho_V = 0.0373$ lbs/ft^3. Then $\rho_L - \rho_V = 59.7627$ lbs/ft$^3 \cong 59.8$ lbs/ft^3, which results in a negligible 0.06% error.)

Consequently,

$$u \cong f \bullet \sqrt{\frac{\rho_L}{\rho_V}}$$

Imposing the equation of continuity: $W = A \bullet \rho_V \bullet u$ or $u = W/A \bullet \rho_V$

Where A = tower cross-section area (ft^2)
ρ_V = vapor density (lbs/ft^3)
u = average vapor velocity in empty tower shell (ft/sec)
and W = vapor mass flow (lbs/sec)

Substituting for u in the equation above:

$$\frac{W}{A \bullet \rho_V} \cong f \bullet \sqrt{\frac{\rho_L}{\rho_V}} \quad \text{then} \quad \frac{W}{A} \cong f \bullet \sqrt{\rho_L \bullet \rho_V} \quad \text{or} \quad A \cong \frac{W}{f \bullet \sqrt{\rho_L \bullet \rho_V}}$$

Use the Ideal Gas Law to express the vapor density.

$$\rho_V = \frac{P \bullet M_{AVG}}{R \bullet T} \quad \text{Then by substitution one obtains} \quad A = \frac{W}{f \bullet \sqrt{\dfrac{P \bullet M_{AVG} \bullet \Psi_L}{R \bullet T}}}$$

Using the Universal Gas Constant R = 0.73 (ft^3)(atm)/(lb-mole)(°R) the equation becomes:

$$A = \frac{W}{1.17 \bullet f \bullet \sqrt{\dfrac{P \bullet M_{AVG} \bullet \Psi_L}{T}}} \quad \text{Now} \quad A = \frac{\pi \bullet D^2}{4} = 0.7854 \bullet D^2$$

and

$$D^2 = \frac{W}{0.9192 \bullet f \bullet \sqrt{\dfrac{P \bullet M_{AVG} \bullet \Psi_L}{T}}} = \frac{1.0879 \bullet W}{f \bullet \sqrt{\dfrac{P \bullet M_{AVG} \bullet \Psi_L}{T}}}$$

Adjusting units: $D = \dfrac{1.043 \bullet 12}{60} \bullet \sqrt{\dfrac{W}{f \bullet \sqrt{\dfrac{P \bullet M_{AVG} \bullet \Psi_L}{T}}}}$ Therefore, $D\,(\text{inches}) = 0.2085 \bullet \sqrt{\dfrac{W}{f \bullet \sqrt{\dfrac{P \bullet M_{AVG} \bullet \Psi_L}{T}}}}$

that liquid entrainment and/or vapor phase pressure drop in the tower will not be excessive.

Excessive vapor velocity will first manifest itself by causing excessive liquid entrainment rising up the tower, causing loss of separation efficiency. Ultimately the excessive entrainment and pressure drop will cause tower flooding.

To achieve a well-balanced tower design, the foregoing analysis must be performed at each stage of the tower, from bottom to top. Composition changes, feed points, draws, etc., each can cause a different requirement. The tower must be examined to locate the limiting point.

Similar analyses, with empirically-observed performance coefficients, are applied to vapor passing through the trays and through the liquid, and to the movement and control of liquid passing through downcomers and across the trays. These analytical procedures are beyond the scope of this text. Reference should be made to the aforementioned text by Robinson and Gilliland for further information.

Considerations in optimizing distillation system design

Optimizing the technical and economic design of distillation equipment and similar gas and vapor/liquid mass transfer systems involves a number of interrelated parameters. The positive/negative balance of a variety of contacting devices with different capacities and efficiencies for promoting vapor/liquid mass transfer must be taken into consideration. Along with the technical issues considered in such designs, economical operation is essential not only in the reduction of energy and other direct costs, but also in relation to investment and return on investment from the operation being considered. In this respect, distillation towers are not independent process-wise, as consideration must also be given to other auxiliaries such as reboilers, condensers, pumps, controls and related equipment.

SIZING TOWERS

In determining optimum diameter and height of towers for distillation, absorption, stripping and similar mass transfer operations, design factors are affected by whether the installations will be indoors or outdoors. With indoor installations, building height limitations, as well as floor level accessibility, are an important factor in the design. Where there are height limitations, towers must be increased in diameter to provide for reduced tray spacing, which in turn will require lower vapor velocities. With outdoor installations, literally the 'sky is the limit', and refinery and petrochemical towers of 200 feet in height are not uncommon.

In either case, indoors or outdoors, the interrelated tower diameter and tray spacing are limited by allowable entrainment factors (*f* factors) (Katzen, 1955). If outdoors, tower heights and diameters must be related to maximum wind loading factors in the specific plant location and may be complicated by allowance for earthquake factors.

TRAY AND PACKING SELECTION

Vapor/liquid contacting devices may be of two distinct types, namely packed or tray (staged) towers. In packed towers, the transfer of material between phases occurs continuously and differentially between vapor and liquid throughout the packed section height. By contrast, in tray towers, the vapor/liquid contact occurs on the individual trays by purposely interrupting down-flowing liquid using downcomers to conduct vapor-disengaged liquid from tray to tray and causing the vapor/liquid contact to occur between cross-flowing liquid on the tray with vapor flowing up through the tray. In other words, the vapor/liquid contact is intermittent from tray to tray, and is therefore referred to as being stagewise. Thus, for any given separation system, the degree of vapor/liquid contact will be greater with a greater height of the packed section, or in the case of tray towers, a greater number of trays used.

It is generally considered that packing-type internals may be used with relatively clean vapor and liquid systems where fouling is not a problem. Economics indicate that packing is applicable in small and modest sized towers. As the towers become larger, packing becomes complicated by the need for multiple liquid redistribution points to avoid potential vapor/liquid bypassing and reduction in efficiency. Structured packings (Fair *et al.*, 1990; Bravo *et al.*, 1985) are designed to minimize these problems by reducing the height requirement and controlling, to some extent, the distribution of liquid. However, high fabrication and specialized installation costs would indicate that these are applicable only for relatively low-volume, high-value product processing.

Trays of various types are predominant in vapor/liquid contacting operations, particularly on the very large scale encountered in the petroleum and petrochemical industries, in large scale operations of the chemical process industries and in the large scale plants of the motor fuel grade ethanol industry.

The venerable bubble cap tray, with a wide variety of cap sizes, designs and arrangements to maximize contact efficiency, has fallen out of favor during the past few decades because of the relatively high cost of manufacture and assembly. Valve trays of several types have taken over in operations requiring a relatively wide vapor handling capacity range (turndown). This has been extended by use of different weights of valves on the same tray. Specialty trays such as the Ripple, Turbogrid, tunnel cap and others designed to improve contact under certain specific circumstances have been used to a limited extent.

The long established perforated tray is a contacting tray into which a large number of regularly oriented and spaced small circular openings have been drilled or punched. These trays are commonly referred to as 'sieve trays' because of the original practice of putting the maximum number of holes in any given tray area. This original design produced a fairly inefficient operation at normal loading, and a very inefficient operation with decreased vapor loading. About 50 years ago, engineers began to suspect that the design approach had been in error, and that the hole area in the trays should be limited by the hole velocity loading factor to obtain maximum contact by frothing, as indicated in Figure 10.

The hole vapor loading factor (or perforation factor) is defined as the vapor velocity through the perforations adjusted by the square root of the vapor density at the specific tower location of a given perforated tray. With the parallel development of separation processes in the petroleum refining, chemical and ethanol industries, the modern approach has developed to what is now called 'perforated tray' design. The Fractionation Research Institute of the American Institute of Chemical Engineers diverted its efforts from bubble cap studies to perforated tray testing; and have established a basis for the design of perforated trays with high efficiency and wide capacity range (Raphael Katzen Associates, 1978).

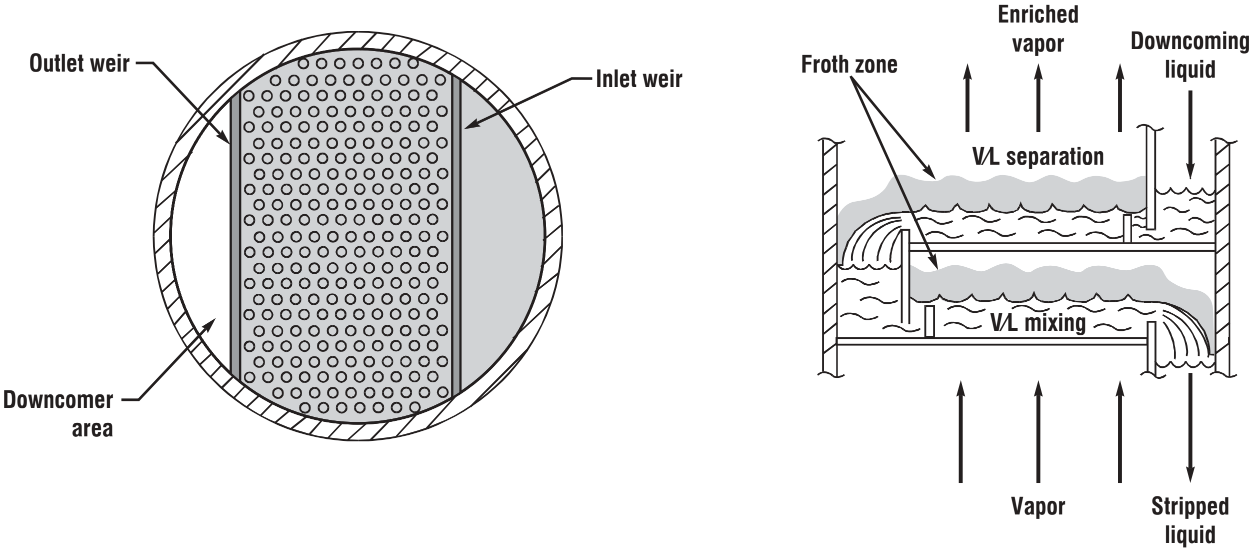


Figure 10. Perforated tray frothing.

Where foaming or tray fouling (caused by deposition of solid materials in the tower feed) can be an operational problem, novel designs such as the baffle tray may permit extended operating time between cleanings. Baffle trays may take a number of different forms. They can be as simple as appropriately spaced, unperforated, horizontal metal sheets covering as much as 50-70% of the tower cross sectional area; or they may take the form of a series of vertically spaced, alternating, solid disc-and-donut rings (see Figure 7). Towers up to 13 ft diameter are in operation using this simple disc-and-donut design concept.

Although system-specific data have been developed for each type of tray, it is difficult to correlate tray loading and efficiency data for a wide variety of trays on a quantitative basis. Each system must be evaluated based upon empirically-derived loading factors for vapor and liquid operations within the tower.

AUXILIARIES

Energy input is of prime importance in tower design, particularly in ethanol stripping and rectification units. In aqueous and azeotrope-forming systems, direct steam injection has been common practice to maintain simplicity. However, the need to reduce the volume of waste going to pollution remediation facilities has minimized use of this simple steam injection technology to avoid the dilution effect of the steam being condensed and added to the stillage. Direct steam injection transfers both the energy and the water into the process. By imposing a heat exchanger (reboiler) between the steam and the process, only the energy is transferred into the tower. The condensate water is returned in a closed loop to the boiler, thus reducing the bottoms outflow from the process. Reboilers are thus growing in acceptance, and several types may be employed. Kettle and thermosyphon reboilers are preferred where fouling is not a problem. Where fouling can occur, high velocity, forced-circulation, flash heating reboilers are preferred. Figure 11 depicts the reboiler energy transfer by a forced-circulation reboiler as compared to Figure 1 which depicts direct steam injection.

Thermocompression injection of steam has also been utilized where low pressure vapors are produced from flash heat recovery installations and where higher pressure motive steam is also available.

Condenser design would appear to be simple. However, in many cases, water limitations require adapting condenser designs to the use

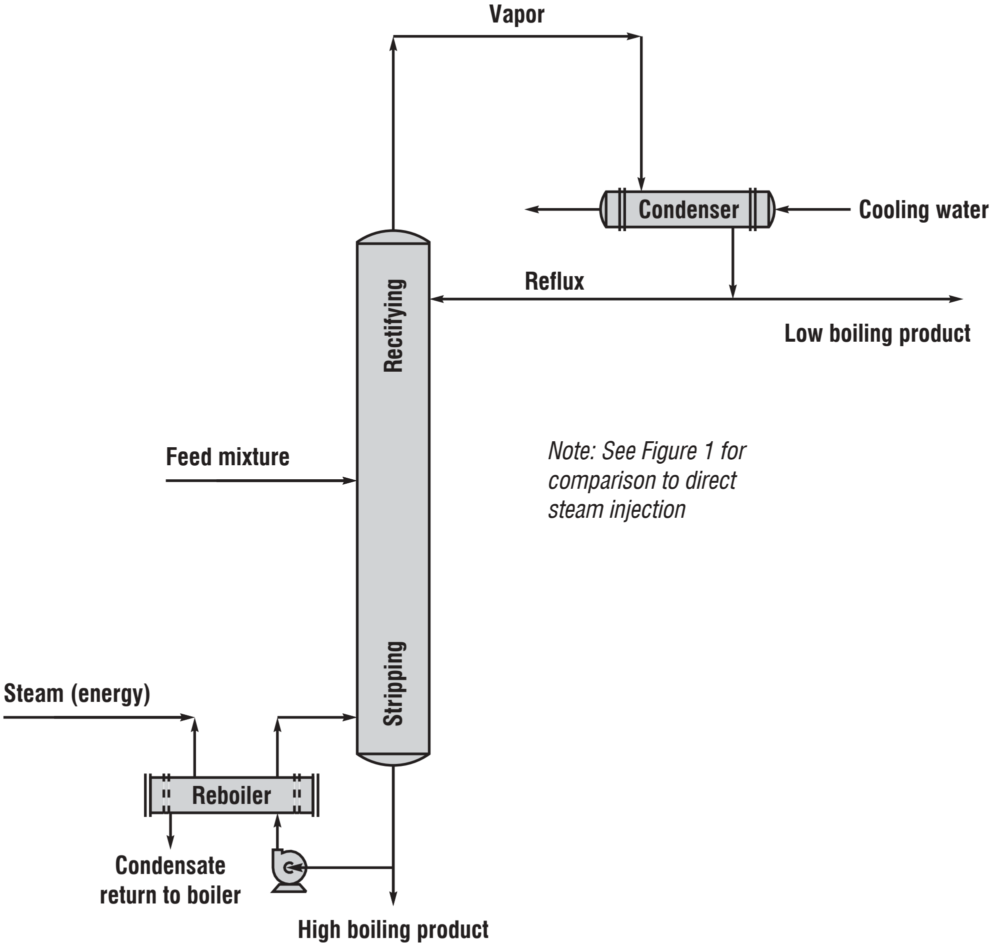

Vapor
Condenser
Cooling water
Reflux
Low boiling product
Rectifying
Feed mixture
Note: See Figure 1 for comparison to direct steam injection
Stripping
Steam (energy)
Reboiler
Condensate return to boiler
High boiling product

Figure 11. Energy transfer by a forced-circulation reboiler.

of cooling tower water with limited temperature rise and minimal scale-forming tendencies. On the other hand, where water is extremely scarce, air-cooled condensers are used.

Energy conservation

The increasing cost of thermal energy, whether provided by natural gas, fuel oil, coal or biomass, is fostering an increased emphasis on heat recovery and a reduction in primary thermal energy usage (Fair, 1977; Petterson *et al.*, 1977; Mix *et al.*, 1978). Conventional bottoms-to-feed heat exchangers are now being supplemented with recovery of overhead vapor latent heat by preheating feed streams and other intermediate process streams. Techniques of multistage distillation (similar to multiple effect evaporation) are also practiced. Pressure-to-atmospheric, atmospheric-to-vacuum, or pressure-to-vacuum tower stages are utilized, with the thermal energy passing overhead from one tower to provide the reboiler heat for the next one. Two such stages are quite common and three stage systems have also been utilized (Katzen, 1980; Lynn *et al.*, 1986).

Furthermore, the modern technique of vapor recompression, commonly used in evaporation systems, is also being applied to distillation systems. Such a system can provide for compression of overhead vapors to a pressure and temperature suitable for use in reboiling a lower pressure stripping tower. However, the compression ratios required for such heat recovery may consume almost as much electrical energy as would be saved in thermal input. Alternative systems, using vapor recompression as an intermediate stage device in the distillation system, have also been proposed.

Control systems

Control systems can vary from manual control, through simple pneumatic control loops to fully automated distributed control (Martin *et al.*, 1970). High level computer control has facilitated the application of sophisticated control algorithms, providing more flexibility, reduced labor and higher efficiency with lower capital investment. Such systems, when properly adapted to a good process design, have proven more user-friendly than the control techniques utilized in the past.

Economic design

In integrating the technology discussed, the final analysis must be economic. Alternative systems must be compared on the basis of investment requirements, recovery efficiency and relative costs of operation. Thus, any heat exchangers installed for heat recovery must show a satisfactory return on the investment involved in their purchase and installation. In comparing alternative separation systems, the overall equipment costs must be compared against energy and other operating costs to determine which system offers the best return. Modern computer-assisted designs incorporate economic evaluation factors so economic optimization can be determined rapidly.

Ethanol distillation/dehydration: specific systems technology

Proven industrial technologies are available for distillation of various grades of ethanol from grain, sugarcane, molasses and other feedstocks. Improvements have been made over the years, particularly during development of the motor fuel grade ethanol industry. In such installations, a key requirement is the minimization of total energy usage.

The operation that has been most subject to critical comment is the distillation process. Many relatively new 'authorities' in the field have based their criticism on technologies that go back 50-60 years, and have created an unwarranted condemnation of distillation as a viable process for low energy motor fuel grade ethanol production. Systems developed over the years will be described to show that much of such criticism is unwarranted and unjustified.

PRODUCTION OF INDUSTRIAL ETHANOL

Prior to the emphasis on motor fuel grade ethanol, the major ethanol product utilized worldwide was high purity, hydrous industrial ethanol, which is generally produced at a strength of 96° GL (192° US proof) (°GL = degress Gay Lussac = % by volume ethanol; US proof = 2 x % by volume ethanol). Efficient systems have been in commercial operation for many years for the production of such high grade ethanol from ethylene, grain, molasses and sulfite waste liquor. The basic distillation system is shown in Figure12.

In the case of synthetic ethanol (outside the scope of this publication), the beer stripping tower is not required and the refining system is a simple three tower unit, which achieves 98% recovery of the ethanol in the crude feed as a first grade product. The final product may contain less than 5 ppm total impurities and has a 'permanganate time' of more than 60 minutes.

For the production of industrial or beverage spirit products made by fermentation of grain, molasses or sulfite liquor, the system utilizes the full complement of equipment shown in Figure 12. The beer feed is preheated from the normal fermentation temperature in several stages, recovering low level and intermediate level heat from effluent streams and vapors in the process. This preheated beer is degassed and fed to the beer stripper, which has stripping trays below the beer feed point and several rectifying trays above it. The condensed high wines from the top of this tower are then fed to the extractive distillation tower, which may operate at a pressure of 6-7 bars (87-101.5 psi). In this tower, most of the impurities are removed and carried overhead to be condensed as a low grade ethanol

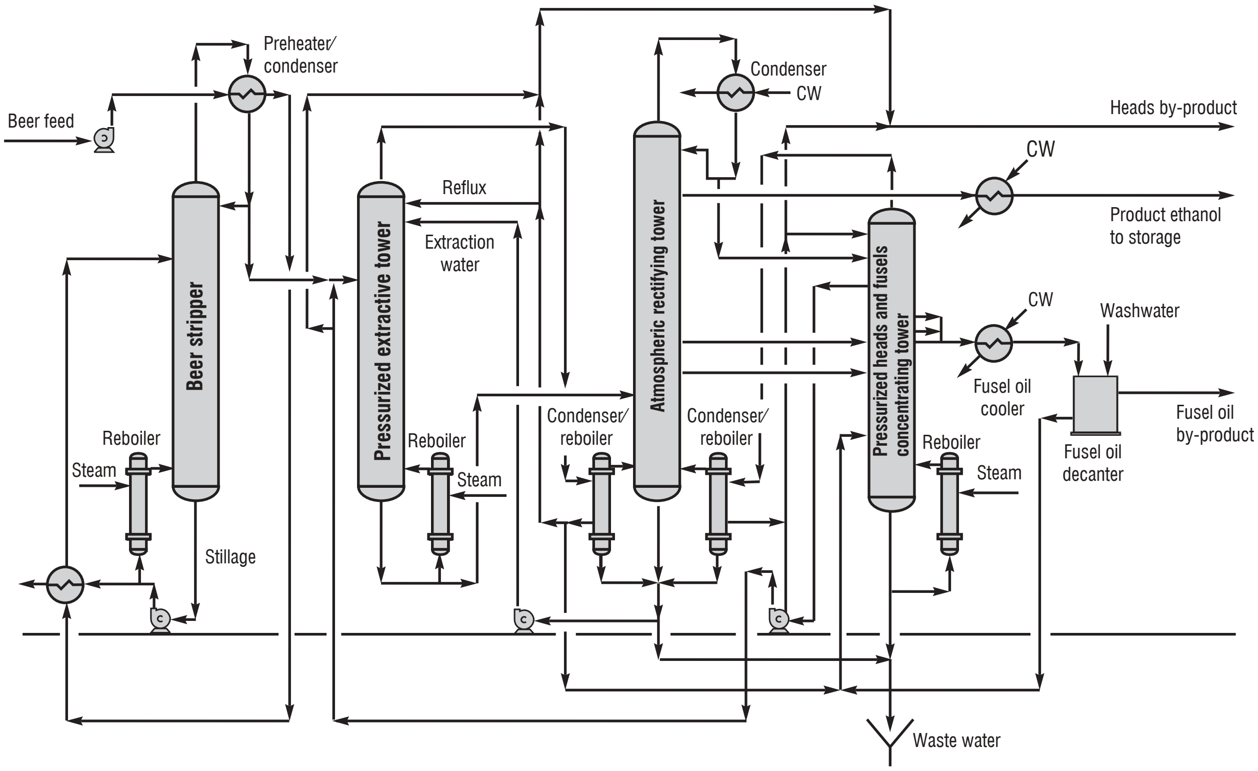

Preheater/
condenser
Beer feed
Beer stripper
Reboiler
Steam
Stillage
Reflux
Extraction
water
Pressurized extractive tower
Reboiler
Steam
Condenser
CW
Atmospheric rectifying tower
Condenser/
reboiler
Condenser/
reboiler
Heads by-product
CW
Product ethanol
to storage
Pressurized heads and fusels
concentrating tower
CW
Washwater
Fusel oil
cooler
Fusel oil
decanter
Fusel oil
by-product
Reboiler
Steam
Waste water

Figure 12. Low energy-consuming high grade hydrous ethanol distillation.

stream, from which a small purge of heads (acetaldehyde and other low boiling impurities) may be taken while the primary condensate flow is fed to the concentrating tower. The purified, diluted ethanol from the bottom of the extractive distillation tower is fed to the rectifying tower, which has an integral stripping section. In this tower, the high grade ethanol product, whether industrial or potable, is taken as a side draw from one of the upper trays. A small heads cut is removed from the overhead condensate. Fusel oils (mixtures of higher alcohols such as propyl, butyl, and amyl alcohols and their isomers, which are fermentation by-products or 'congeners') are drawn off at two points above the feed tray but below the product draw tray to avoid a buildup of fusel oil impurities in the rectifying tower. The overhead heads cut and the fusel oil draws are also sent to the concentrating tower.

It should be noted that the rectifying tower is heated by vapors from both the pressurized extractive distillation tower and the pressurized concentrating tower.

In the concentrating tower, the various streams of congener-containing draws are concentrated. A small heads draw is taken from the overhead condensate, which contains the acetaldehyde fraction along with a small amount of the ethanol produced. This may be sold as a by-product or burned as fuel. A fusel oil side draw is taken at high fusel oil concentrations through a cooler to a washer. In the washer, water is utilized to separate the ethanol from the fusel oil, with the washings being recycled to the concentrating tower. The decanted fusel oil may be sold as a by-product. The ethanol recovered from the crude streams is taken as a side draw from the concentrating tower and fed back to the extractive distillation tower for re-purification and recovery of its ethanol content.

In an early version of this system, installed more than 60 years ago for the production of potable ethanol from grain and from molasses, all towers were operated at atmospheric pressure. However, installations made within the past 40 years utilize the multistage pressure system to reduce energy consumption to a level of about 50% of the all-atmospheric system.

The commercial installations utilizing the multistage pressure, or 'pressure cascading' technique operate with a steam consumption of 3.0-4.2 kg of steam/liter (25-35 lb/gallon) of 96º GL ethanol. This may be compared to about 6 kg of steam/liter for earlier conventional distillation systems.

Production of anhydrous ethanol

Systems have been designed and installed for production of extremely dry and very pure anhydrous ethanol for food and pharmaceutical use, primarily in aerosol preparations. These systems, as shown in Figure 13, yield ethanol containing less than 200 ppm water (99.98º GL), less than 5 ppm total impurities and more than 60 minutes permanganate time.

The two tower dehydrating system has been operated in two super-anhydrous plants in Canada, and was used to produce motor fuel grade ethanol (99.5º GL) in four installations in Cuba (prior to the advent of the Castro regime). The dehydrating tower and the entrainer-recovery tower are operated at atmospheric pressure. Thus, they may utilize either low pressure steam, hot condensate or hot waste streams from other parts of the ethanol process to minimize steam usage. To simplify equipment and minimize investment, a common condensing and decanting system is used for the two towers.

The entrainer used to remove water as a ternary (three component) azeotrope may be benzene, heptane (C_6-C_8 cut), cyclohexane, n-pentane, diethyl ether or other suitable azeotropic agents. The entrainer serves to create a three component azeotrope that boils at a temperature lower than any of the three individual components and lower than the ethanol/water binary (two component) azeotrope. Therefore the ternary mixture will pass overhead from the tower, carrying the water upward. Upon condensing, the mixture separates in a decanter into an entrainer-rich layer and a water-rich layer.

The hydrous ethanol feed enters the dehydrating tower near the top. The feed contacts the entrainer in the upper section of the tower. The three component mixture in this section of the tower seeks to form its azeotrope, but is deficient in water and contains more ethanol than the azeotrope composition. Therefore, the ethanol is rejected downward in the liquid and is withdrawn as an anhydrous product from the bottom of the tower. The water joins the entrainer, passing upward as vapor to form a mixture that is near the azeotrope

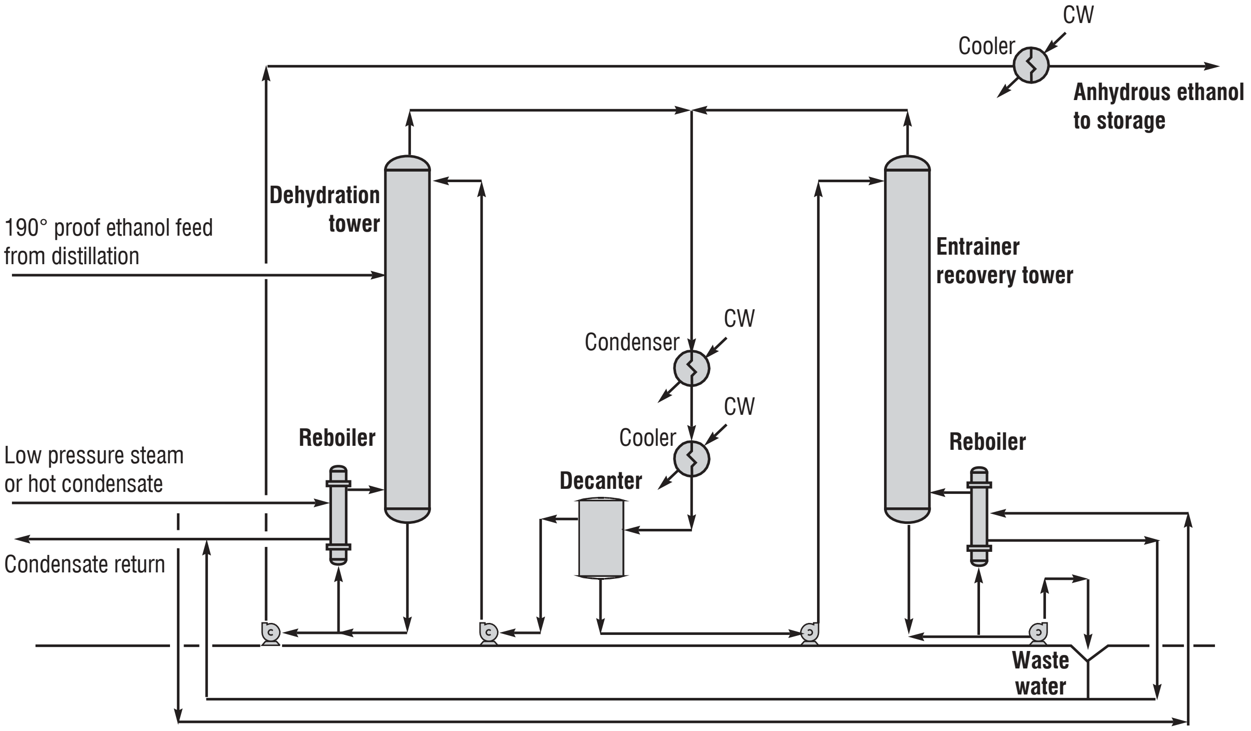


Figure 13. High grade anhydrous ethanol system.

composition for the three components. The condensed mixture separates into two layers in the decanter and the entrainer-rich layer is refluxed from the decanter back to the top of the tower. The aqueous layer is pumped from the decanter to the entrainer-recovery tower, in which the entrainer and ethanol are concentrated overhead in the condenser-decanter system. The stripped water, emerging from the base of the tower, may go to waste. If it has substantial ethanol content, it may be recycled to the spirit unit, but this introduces the risk of traces of the entrainer in the hydrous ethanol which may not all be sent to the dehydration system. This system operates with a steam consumption of 1-1.5 kg/liter (8.3-12.5 lb/gallon) of anhydrous ethanol depending on the quality of product required. As indicated above, a major part of the equivalent steam energy can be provided by hot condensate and hot waste streams from the spirit unit.

References

Bravo, J.L. *et al.* 1985. Hydrocarbon Proc. Jan. p.91.

Katzen, R. 1955. Chem. Eng. Nov. p. 209.

Fair, J.R. 1977. Chem. Eng. Prog. Nov. p. 78.

Fair, J.R. *et al.* 1990. Chem. Eng. Prog. Jan. p. 19.

Katzen, R. 1980. Low energy distillation systems. Bio-Energy Conference, Atlanta, GA, April.

Lynn, S. *et al.* 1986. Ind. & Eng. Chem. 25:936.

Martin, R. L. *et al.* 1970. Hydrocarbon Proc. March 1970, p. 149.

Mix, T.J. *et al.* 1978. Chem. Eng. Prog. April p. 49.

Petterson, W.C. *et al.* 1977. Chem. Eng. Sept. p. 79.

Raphael Katzen Associates. 1978. Grain Motor Fuel Alcohol Technical and Economic Assessment Study. Prepared for: US Department of Energy HCG/J6639-01.

Robinson and Gilliland. 1950. Elements of Fractional Distillation, McGraw Hill Book Co., Inc.

Chapter 23

Development and operation of the molecular sieve: an industry standard

R.L. BIBB SWAIN

Delta-T Corporation, Williamsburg, Virginia, USA

Introduction: the early days

AZEOTROPIC DISTILLATION

The equipment used during the dawn of the fuel ethanol program was built using a strange combination of technologies borrowed from different industries. Most of the basic production expertise came from the beverage alcohol industry, but there was no need to remove all the water from beverage alcohol. To prevent phase separation when blended with gasoline, fuel grade ethanol needed to be almost completely dry. Standard distillation still leaves over 4% water in the ethanol, so a process called 'azeotropic distillation' was used in all the early commercial ethanol plants to remove the final water from (dehydrate) the ethanol. Azeotropic distillation uses a third component, typically benzene or cyclohexane, to 'break' the azeotrope (the composition above which standard distillation becomes ineffective).

Azeotropic distillation systems tended to be quite expensive, difficult to operate and adjust, and consumed a significant amount of energy. Attempts were made to reduce the operating difficulties and energy consumption, but the two goals were incompatible. Multi-pressure techniques could reduce energy consumption, but at the expense of more difficult operation and considerably higher capital cost. A process upset could easily contaminate the ethanol product with benzene; and there was no way to totally protect plant workers from exposure to known or expected carcinogens.

SYNTHETIC ZEOLITES AND EARLY MOLECULAR SIEVE DEHYDRATORS

An inventor named Skarstrom received a patent in 1957 for a device that utilized a synthetic zeolite adsorbent that selectively removed water from air and some other gasses and vapors (Ruthven, 1984). A different zeolite could even be used in virtually the same device to separate oxygen and nitrogen from air (Ruthven, 1984). These synthetic zeolites became known as 'molecular sieves' due to the very precise pore size that enabled them to select and remove one molecule size from a bulk mixture containing molecules with a larger size or lower polarity. A properly designed system could dry air to a dew point of -100ºF; and drying air became the first major application for molecular sieves. By the early 1980s, at least a dozen companies offered molecular sieve air dryers.

If molecular sieves could be used to dry air, why not ethanol? Experiments were under way at that time by several inventors and companies to prove efficacy of that concept. In the August 1981 issue of *Gasohol USA* (the only trade journal at the time), two companies advertised molecular sieve dehydrators that dried ethanol in the vapor phase. Ad-Pro Industries, Inc. of Houston, Texas, and Anhydrous Technology, Inc. from Sugarland, Texas, both offered small skid-mounted systems for drying up to 200 gallons per hour. Two other companies, Pall Pneumatics and Shroeder Farms, were marketing molecular sieve ethanol dehydrators that dried the ethanol in the liquid phase. The liquid phase systems used too much energy, had severe problems with desiccant fouling and finally were just not able to compete with the vapor phase units. The two companies eventually sold approximately a dozen of the small vapor phase dehydrators. Since the devices were relatively simple, many entrepreneurs built unauthorized copies for themselves and others.

The early vapor phase dehydrators had problems of their own. Aside from a general lack of reliability, the molecular sieve desiccant would deteriorate within a year; and all the dust that resulted would contaminate the ethanol product. The commercial molecular sieve desiccant was manufactured by mixing the molecular sieve crystalline powder (much like glass dust) with a clay binder, and forming the slurry into beads or extruded pellets about 1/8 inch in diameter. The finished product had the look and toughness of limestone. Any movement of a bed of the material resulted in abrasion between the particles. The resulting dust accumulated in the bed causing vapor channeling and product contamination. Bed movement could be caused by excessive temperature swings (thermal expansion and contraction), excessive pressure drop across the bed (fluidization) and local disturbance by high-speed vapor entering the bed during cycle transition (jetting). By 1987, Ad-Pro Industries and Anhydrous Technology, whose designs were subject to all these problems, were both out of business.

NEW TECHNOLOGY BECOMES PROVEN ON LARGE SCALE

In 1984, Delta-T Corporation was formed in Williamsburg, Virginia, to build and market a molecular sieve dehydrator design that had been developed over the past three years by Delta-T's founder. The new design eliminated the reliability and desiccant deterioration problems that plagued earlier designs. Delta-T built a 150-gallon/hr prototype at a small ethanol plant in Charles City, Virginia. After conducting a battery of tests to prove the concept and optimize the design, the prototype continued to produce ethanol commercially at the plant until the Virginia ethanol incentive expired many years later. Delta-T sold six dehydrators during its first eight months of operation, the largest of which was 800 gallons/hr.

In the meantime, larger ethanol plants continued to be built using azeotropic distillation dehydration technology. Molecular sieve dehydration was a relatively new technology with a somewhat troubled past; and Delta-T had no large reference unit to show a customer. In addition, the firms providing technology for the balance of the plant had their own azeotropic distillation technologies to sell the customer. They were quick to point out both the difficulties of getting uniform vapor flow through large beds and that the technology was not proven at a larger scale. Both were correct, but misleading.

Not able to sell a large dehydrator to an American ethanol plant, Delta-T formed a joint-venture with an Italian engineering firm to build a 30 million gallon/year dehydrator for the largest distillery in Europe, located in Sicily. Design specifications called for the feedstock ethanol to be as low as 160 proof (80% ethanol by volume), with a 199.4 minimum product proof. Performance guarantees were stringent, but Delta-T badly needed a large reference unit, so it contracted to provide the unit. Happily for all project participants, the new world-scale dehydrator worked just as promised, and Delta-T finally had proven the design at large scale.

Molecular sieve dehydrators had always offered the industry lower capital costs, lower operating costs, and greater personnel safety than azeotropic distillation. Now that a large unit was on line and performing well, the last obstacle to industry acceptance was overcome. At first, ethanol plant developers would get their plant technology from the older process design firms, but they would come directly to Delta-T for their molecular sieve dehydrator. When the process technology firms realized they would no longer be able to sell azeotropic distillation dehydration to their customers, they reluctantly adopted molecular sieve technology. Today, all new ethanol plants are built with molecular sieve dehydrators.

How does a molecular sieve dehydrator work?

PRESSURE SWING ADSORPTION

Most modern molecular sieve dehydrators use a process known in the industry as 'pressure swing adsorption' to remove water from a vaporized feed stream. We will concentrate herein on the removal of water from an ethanol vapor stream, but many other applications are possible with virtually the same device. The term 'pressure swing' refers to the fact that the dehydrator uses a relatively high pressure when water is being removed from the feed stream and a relatively low pressure when the molecular sieve desiccant is being regenerated (having water removed from the desiccant). Most commercial designs have two or more beds of desiccant and cycle the vapor flow through the beds to provide continuous operation. While one bed is on-line drying the feed vapor, another bed is being regenerated. In some designs one or more additional beds are being depressurized or repressurized in preparation for the next cycle (Le Van, 1996).

HOW DOES ADSORPTION WORK?

The 'macroscopic' principles of adsorption are quite simple, but attempts to accurately explain how adsorption works often fail because the author gets bogged down in technical jargon. The basic characteristic of an adsorbent material is a stronger affinity for one type of atom or molecule than for the other types in the vapor stream. In the case of a molecular sieve ethanol dehydrator, we select an adsorbent with a strong affinity for water and little affinity for ethanol and the other impurities typically contained in the ethanol feed stream. As wet ethanol vapor passes through the bed, the desiccant adsorbs the water molecules but not the ethanol molecules. This process cannot continue indefinitely, because the desiccant has a finite capacity for water; and that capacity is affected by the operating temperature and pressure. Moisture capacity increases as pressure increases or temperature decreases, and vice versa. Since a pressure swing adsorption dehydrator operates at almost constant temperature (Ruthven, 1994), we can desorb the water adsorbed on the previous cycle by lowering the operating pressure and passing a purge vapor through the bed in the opposite direction to sweep the water molecules from the free space surrounding the desiccant beads.

The class of materials known as zeolites occurs naturally, but most commercial molecular sieves are man-made. Synthetic zeolites have a crystalline lattice structure that contains openings (pores) of a precise size, usually measured in angstroms (Å). Molecular sieves can be manufactured with different pore sizes by using different chemistry and manufacturing methods. A synthetic zeolite of type 3Å is used in most ethanol dehydrators, because the pores are 3Å in diameter while water molecules are 2.8Å and ethanol molecules are 4.4Å. Therefore, water molecules are strongly attracted into the pores but ethanol molecules are excluded. Many other adsorbent materials are available that have an affinity for water such as activated alumina. However, other adsorbents have a wide range of pore sizes and therefore are much less selective than molecular sieves.

Water is so strongly attracted to type 3Å molecular sieve that for each pound adsorbed, 1,800 BTUs of heat are released. This effect is referred to as the heat of adsorption. When you remove that same pound of water during regeneration, you must supply 1,800 BTUs of heat (referred to as the heat of desorption). Thus, as feed vapor is dehydrated the bed of desiccant heats up; and as the bed is regenerated it cools down. Type 3Å molecular sieve is capable of adsorbing up to 22% of its weight in water, but pressure swing adsorption dehydrators operate at a much lower moisture loading and limit the dehydration period to prevent an excessive temperature swing.

Operational considerations

ENERGY NEEDS, PRODUCTION CAPACITIES

By limiting the adsorption period, temperature swings are kept to a reasonable value and the heat of adsorption can be effectively stored in the bed of desiccant as sensible heat. The heat is then available to supply the necessary heat of desorption during the regeneration period. The ability to recover the heat of adsorption is why molecular sieve dehydrators are so energy efficient. Assuming the feed vapor comes directly from the rectifier column, the only energy used directly by the molecular sieve dehydrator is the steam required to superheat the vapor to bed operating temperatures (0.1 to 0.2 lbs of steam per gallon of anhydrous ethanol) and the electricity to operate the pumps (0.02-0.03 Kw hr per gallon). Many companies forget that additional energy is required to redistill the liquid that results during regeneration as well as the electricity to operate the cooling tower and air compressor. A properly designed system will have a totally inclusive energy consumption of about 4,000 BTUs per gallon of anhydrous ethanol, assuming the feed ethanol contains 5% water and the product is dried to 0.25% water. Additional energy is required if the feed ethanol is wetter or if the product is dryer.

Molecular sieve dehydrators can accommodate a wide range of specifications, and can be built for any desired production rate. Some are currently in service drying ethanol containing as much as 20% water and some can produce anhydrous ethanol with as little as 20 ppm water in the final product. The smallest units are designed for a product rate of about two gallons per hour (mostly for lab and test work), while the largest can produce 100 million gallons per year.

A TYPICAL OPERATING CYCLE DESCRIBED

The following describes a typical operating cycle for a 2-bed dehydrator drying ethanol containing 5% water. We begin with Bed 1 on-line (drying the feed vapor) and Bed 2 being regenerated (see Figure 1). Wet ethanol vapor enters the top of Bed 1 under a modest pressure, passes downward through the bed and exits the bottom as dry vapor. 60-85% of the vapor leaves the system as anhydrous ethanol product; and the

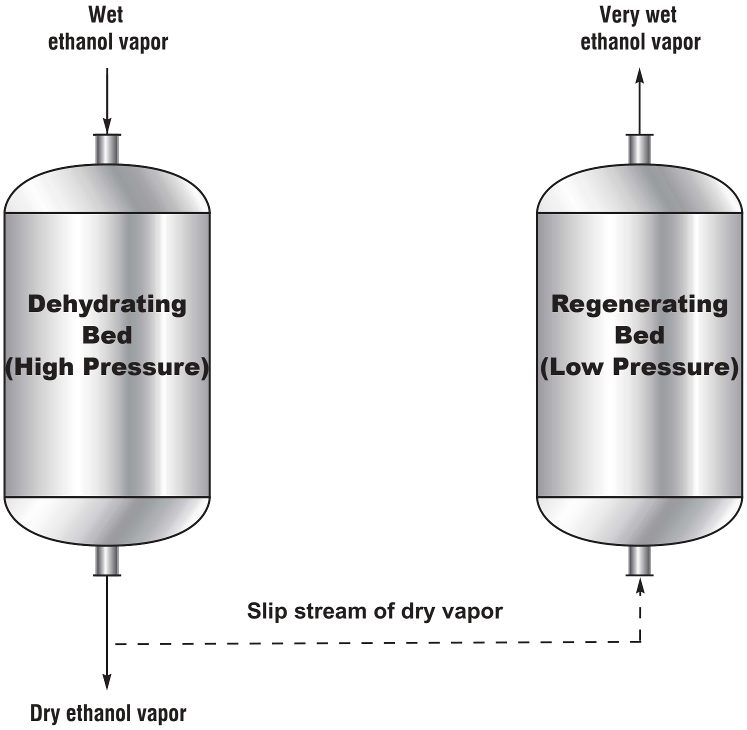


Figure 1. Operation of two bed molecular sieve dehydrator.

remainder is fed to Bed 2 as a regeneration purge stream. Bed 2 is maintained under a vacuum that shifts the equilibrium conditions so that the adsorbed water is desorbed. Operating pressures, temperatures and flow rates are adjusted so that regeneration is complete at the point in the cycle when the beds change position (valves are positioned to reverse the roles of the two beds). One bed typically stays on-line dehydrating the feed stream for 3-10 minutes before being regenerated. When the beds transition from dehydrate to regenerate mode, the pressure in the inlet header tends to sag, which can upset the rectifier column if provisions are not made to mitigate the pressure drop. After the beds switch, the bed that was being regenerated 'pressures up' to the dehydrating pressure and the vaccuum system lowers the pressure in the other bed to the regenerating pressure. The cycle repeats itself continuously. Since one bed or the other is always receiving and drying the inlet vapor, the process seems to be continuous; but in reality it is more properly termed as 'cyclic batch'.

While a molecular sieve dehydrator can operate on a simple timed cycle, the best systems are managed by an 'intelligent' controller (computer or PLC) to provide greater operating safety, reliability and energy efficiency. Once adjusted to standard operating conditions, the molecular sieve dehydrator requires little operator interface and can tolerate reasonable variations in feed rate or quality without need for readjustment. Typical quality control measures include sampling the product, feed and regeneration liquid at regular intervals to assure the product meets specifications and that the system is operating efficiently.

References

LeVan, M.D. 1996. Fundamentals of adsorption. Proceedings of the 5th International Conference on Fundamentals of Adsorption. Kluwer Academic Publishers, Boston, MA.

Ruthven, D.M. 1984. *Principles of adsorption and adsorption processes.* John Wiley & Sons, New York.

Ruthven, D.M., S. Farooq and K.S. Knaebel. 1994. *Pressure swing adsorption.* VCH Publishers, Inc., New York.

Engineering ethanol fermentations

Chapter 24

Water reuse in fuel alcohol plants: effect on fermentation
Is a 'zero discharge' concept attainable?

W.M. INGLEDEW

Applied Microbiology and Food Science Department, University of Saskatchewan, Saskatoon, Saskatchewan, Canada

The subject of water relationships in a fuel alcohol distillery is an interesting and complex issue. Stringent emission controls are in part forcing engineers to design and run newer distilleries to be virtually free of organics in stack emissions and in any water effluents (Bryan, 2003). This dictates that most water outputs in the distillery (except for the cooling tower waters) are recycled inside the plant, and that organic materials in those waters are therefore not dispersed onto fields or into water bodies where they have a BOD (biological oxygen demand) or COD (chemical oxygen demand) impact on the surrounding environment. 'Zero discharge' is a laudable goal in this industry, but such design schemes have created significant problems for microbiological fermentation. Incomplete understanding of such issues led to a comment I first made at the 1999 Fuel Alcohol Workshop: "*A fermentor is not a garbage can*". This comment, made due to concerns in certain fuel alcohol plants, became a catalyst for discussions among engineers, biochemists and microbiologists associated with this multidisciplinary industry, and is beginning to lead to extensive analyses of water effluents from all conceivable sites in modern alcohol plants.

The pre-cook section of a generic dry milling fuel alcohol plant is given in Figure 1. It is in this area or at the fermentor that waters are reincorporated into the new mash. In Figure 1, input water entering the system via the grain is relatively easy to quantitate, as the average moisture levels of corn or wheat are normally known. Well water (part of the makeup water used in the cook), recycled water such as backset, carbon dioxide scrubber water, side stripper bottoms recycle water, and methanator recycle water, are all included. Some of the volatile compounds in these streams, ethanol included, would be lost in cooking due to the temperature employed and vented in part into the atmosphere. However, a significant portion of the ethanol remains in the pressurized slurry during the cook and hold cycle, and then flashes off. The energy and steam vapor content is used to run the distillation columns.

It should be noted that most of the existing plants do not incorporate biomethanator technology, and therefore evaporator condensate water is added directly to the cook water tank. When biomethanation is accomplished, a side discharge line is made available for removal of some of the water from the plant when/if necessary. If methanator water is discharged, well water volumes are increased correspondingly. Minor input streams of much less concern in the plant include addition of enzymes (α-amylase at the mingler and at liquefaction; glucoamylase and urea at the fermentor; or ammonia/ ammonium hydroxide at the mingler. Yeast and

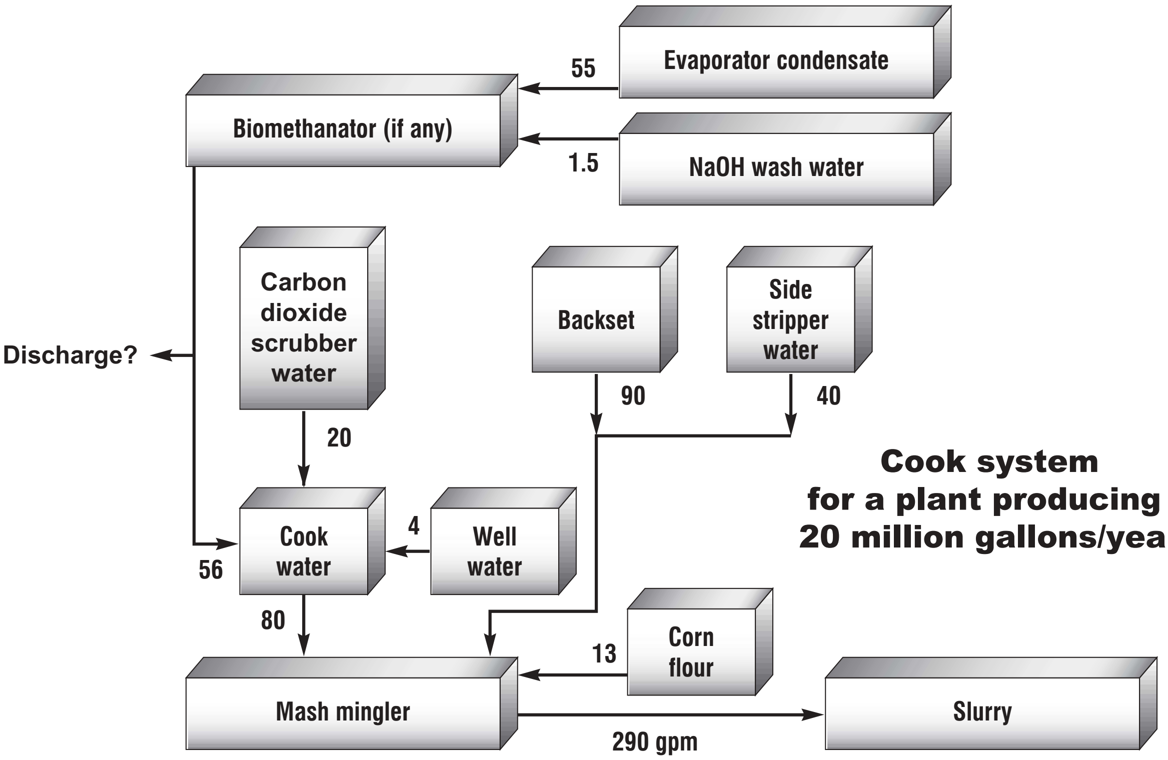

Evaporator condensate
55
Biomethanator (if any)
1.5
NaOH wash water
Carbon dioxide scrubber water
Backset
Side stripper water
Discharge?
90
40
20
Cook system for a plant producing 20 million gallons/year
Cook water
4
Well water
56
80
Corn flour
13
Mash mingler
Slurry
290 gpm

Figure 1. Water inputs and outputs from the pre-cook system of a typical dry mill plant (numbers are in US gallons per minute).

yeast nutrients are added at the fermentor. An approximate mash bill in a dry mill plant is given in Table 1.

Table 1. An approximate mash bill for 50 tons/day of mash in a dry mill fuel alcohol plant.

Component in mash	*Tons of water*
Grain 13.2 tons @ 15%	2.0
Plant water	5.3*
Evaporator condensate	15.9*
Flash tank condensate	1.4*
Steam	~1.2*
Calcium chloride	0.03
Wash waters with NaOH etc.	Volume not determined*
Backset	12.9*
Ammonia	0.06
Yeast	Variable**

*Components that should be monitored for chemical content.
**Components that should be monitored for microbial content.

Output water is more difficult to quantitate. Wet distillers grain, distillers modified wet grains (DMWG) and distillers dried grains with solubles (DDGS) contribute water output values in relation to the degree of drying of the solids. Less well quantified is stack moisture resulting from drying of the grain. Other outputs include evaporator syrup (if marketed separately from wet or dried grains), dryer water emissions, and stack emissions.

The theme of this chapter is that substrate is continually used and ethanol and carbon dioxide are continually produced in the system whether it operates by batch or continuous technologies. All other components, either unused in the medium or made by the yeasts (or by contaminants in the fermentation) are recycled. Some waters that recycle are continually increasing in concentration of the compounds present until the most inhibitory of these eventually affects fermentation rate by slowing the growth rate of the yeast, killing the cells or altering yeast metabolism. This leads to loss of ethanol concentration, reduced yields, sugar (substrate) 'bleed' into the beer well and a general lack of performance of the plant. It should be noted that corn wet mill plants – for example those that use starch slurry, corn steep liquor and backset – have the same general considerations as dry mill plants. A complete and accurate audit of water in and water out will, however, have to await a thorough examination of all systems in selected facilities that can then be used as models.

In most plants, the volumes of water, water-based chemicals and the water contents of by-products can be read off computer screens or estimated by the weight of the products and analytical data. What these plants lack, however, is an exact quantitation on composition of these streams, and the variability of contents and volumes over time, as well as information on special purges of water such as seen with sodium hydroxide cleaning solutions or sanitizers. A major concern with process input streams is toxic or inhibitory components of the streams that affect yeast and therefore the rate and extent of ethanol manufacture. Sodium ion and other components will be discussed later in this context. These considerations put in the context of continuous fermentation systems are even more problematic. Purges of waste streams of undesirable content into fermentors can create an upset to continuous fermentation trains, causing cascading changes from the upstream site of entry throughout the rest of the train. Moreover, the maximum concentration of such chemicals is always in the fermentor with the most crucial need for ideal conditions – the first fermentor (or pre-fermentor) – where growth of yeast is an absolute requirement. More than a week may be required to regain the balanced growth conditions characteristic of continuous fermentation. It is no wonder then that some plants never really operate in a constant or consistent mode (in balanced growth) for any extended time!

Some of the input components with potential problems for fermentation are listed and detailed below. Compositions and variabilities of the components found in each recycled source are all not yet well understood.

Light corn steep water

A representative composition of steep water from the wet milling of corn is recorded in Table 2. Steep water is used as the primary source of nutrients in fermentation of the ~40% starch slurry obtained as a by-product in corn wet milling plants. Although used only once in theory, steepwater residues recycle in thin stillage or backset, and this thin stillage therefore contains all components that were not used by yeast or that were produced in fermentation but not distilled out of the system. During each recycle, the concentrations of the materials recycled will increase. Note that Table 2 does not provide ion analyses.

Table 2. Composition of light steep water.

Characteristics	
pH	3.98
Specific gravity (density meter)	1.0527 (13.7 g/100 mL)
Dry weight, g/100 mL	11.8
N (Kjeldahl method), g/100 mL	0.80
α-amino N, mg/L	756*
Total amino N, mg/L	1035*
Carbohydrate, g/100 mL	5.0*
Lactic acid, g/100 mL	3.82*
Acetic acid, g/100 mL	0.21*

*HPLC analysis
Thomas and Ingledew (unpublished data)

Table 3 provides an estimate of the amino acid content in unused corn steep liquor, and therefore the value of this component in the nutrition of yeast. Yeasts only use amino acids, small peptides (<3 amino acids long), ammonium ion, and urea as nitrogen sources. The free amino acids are preferentially utilized, but in a distinct order.

Table 3. Free amino acids in light steep water.

α-amino acid nitrogen (μmole/mL)			
Asp	0.93	Tyr	0.94
Glu	1.57	Val	3.91
Ser	5.22	Met	1.90
Gly	2.62	Cys	0.19
His	0.73	Ile	1.98
Thr	4.03	Leu	8.5
Ala	11.68	Phe	3.08
Arg	2.03	Trp	0.57
Pro	nd	Orn	1.38
		Lys	2.74

Thomas and Ingledew (unpublished data)

These data show that the amino acid spectrum in this light steep water is broad and varied, providing good amino acid nutrition to the mash in which it is used (note that a major dilution of all compounds would be made on addition of the corn steep water to the starch slurry used in wet mills to make the mash). Steepwater is normally used at about 12% of the mash volume (Table 4).

Table 4. Approximate makeup of mash in a 1000 gpm mash continuous fuel alcohol distillery, showing major input waters and the ~40% starch slurry.

Components	*Gallons per minute*
Starch slurry	252
Stillage	415*
Stripper bottoms	68*
Steepwater	123*
Enzymes, ammonia or urea, soda ash	5
Well water	138*
Yeast (depends on balance of the process)	??**

*Components that should be monitored for chemical content.
**Components that should be monitored for microbial content.

Usable nitrogen is deficient in all grain samples that could possibly be used for fuel alcohol production worldwide (Table 5). Nitrogen deficiency is a main cause of stuck and sluggish fermentations in this industry. For this reason, virtually all fuel alcohol plants add liquid or solid urea, ammonia, or ammonium hydroxide, although the amounts may not be optimized.

Table 5. FAN levels in typical mashes from grains usable for fuel alcohol production.

Grain	*Free amino nitrogen (mg/L mash*)*	
	Total FAN	*Usable FAN*
Wheat	82	64
Barley	84	62
Hulless barley	124	100
Oats	193	159
Hulless oats	184	130
Rye	103	83
Molasses	267	141
Corn	70	58
Starch slurry	~ 0	~ 0

*All mashes normalized to 22% total solids for comparison. Thomas and Ingledew, unpublished data.

Addition of extra usable nitrogen has been shown to improve the growth of yeast and the rate of fermentation of mashes (Ingledew, 1995; 1999). More importantly, higher concentrations of alcohol can then be made. This is the basic principal behind very high gravity (VHG) fermentation technology (developed in this lab) being used today in the fuel alcohol industry where concentrations of ethanol well above 16% v/v can now be made under routine conditions in a fuel alcohol plant.

The quantity of lactic acid in corn steepwater is noteworthy. It originates from the bacteria that are believed to play an important part in the wet milling of corn – bacteria which grow and metabolize during the steeping process. After some recycling of thin stillage through sequential fermentations, the lactic acid levels can become significant, inhibiting yeast growth and metabolism (Narendranath *et al.,* 1997). Lactic acid content limits the amount of corn steep liquor (and perhaps also thin stillage) possible in fermentation mash makeup, as do the foaming properties of these streams (leading to entrainment of liquid in the carbon dioxide environment in the headspace), which has been known to cause 'blowing' of pressure relief valves or plates on the tops of fermentors, creating an immediate and dangerous carbon dioxide problem for personnel in the plant.

Thin stillage

Thin stillage is used in both dry and wet mill plants. Although it varies from plant to plant, it is generally low in utilizable nitrogen compounds and in total nitrogen. In fact, there are few, if any, positive reasons (other than water recycling) to use thin stillage. Table 6 provides an analysis of thin stillage along with a grouped estimate of the major minerals in the corn (P, S, K, Ca, Mg, Cu, Fe, Mn, and Zn only). Phosphorus levels ranged from 815 to 1762 mg/L while potassium ranged from 705 to 2643 and magnesium levels in all stillages examined were 200-721 ppm. Other elements were much lower, including Zn, Fe, Cu, Mn. Sodium ion was not measured at that time.

The lactic acid concentration in a wet mill alcohol plant can also be problematic. In these plants, it can be shown that the concentration of lactic acid contributed by the light steep water and the stillage can easily exceed the 0.8% (8 g/L) minimal concentration reported to affect yeast (Narendranath *et al.,* 1997; 2001a; 2001b). In this respect, the valuable contribution made to yeast nutrition by light steep water is somewhat negated by the lactic acid present in it and in thin stillage – acid which inhibits the growth and metabolism of yeast. The extent of this contribution is shown in Table 7.

Table 6. Proximate composition of corn thin stillage.

Components	*g/L*
Total solids	48
Total nitrogen	0.53
Crude protein	3.0
Free amino nitrogen (mg/L)	75
Glucose	0.60
Lactic acid	11
pH	3.7
Minerals	3.64

Jones and Ingledew, 1994

Table 7. Lactic acid concentrations in fermentors in wet mills entering the system from stillage and steep water.

Mash constituent	*L/1000 L mash*	*Lactic acid (g/L)*	*Final mash concentration (g/L)*
Light steep water	134	38.2	4.7
Stillage	< 415	11.0	4.57
Total			9.27

Again, a complete audit of water composition (including recycled waters) in all areas of the distillery would allow us to determine the exact influence that each water stream (with its own particular chemical composition) might have on the fermentation process. As not all of the above materials have been repeatedly analyzed over a broad range of times and conditions so that the significance of their contents is known, this chapter can only discuss what we do know about some of the water components.

A few years ago, we analyzed six samples of backset from batch fermentation plants. Half the samples were from wheat plants and half from corn plants. These results are shown in Table 8 and were published by Jones and Ingledew, 1994.

Table 8. Backset samples from six fuel alcohol plants.

Constituent	*Wheat*	*Wheat*	*Wheat*	*Corn*	*Corn*	*Corn*
Total solids, % w/v						
Solubles	4.6	6.3	3.4	4.5	2.8	2.4
Insolubles	8.0	7.8	2.3	3.2	2.0	2.0
Total N, % w/v	0.18	0.11	0.13	0.03	0.05	0.54
FAN, mg/L	96	79	51	118	75	88
Glucose, mg/mL	1.6	33	1.0	0.7	0.6	0.2
Lactic acid, g/L	11	17	1.5	7	11	6
pH	4.2	3.6	4.7	4.7	3.7	4.0
Minerals, mg/L	5648	2085	2893	4149	3626	2946

A number of differences were noted among these six samples chosen at random from industry. Note the levels of lactic acid, which we know begin to exert a strong effect on yeast at 8 g/L. Note also that the free amino nitrogen values (FAN) are quite low, because any usable FAN in the stillage had already been made available to the yeast in one or more prior fermentations. FAN is a measure of the free amino groups in amino acids, small and large peptides and soluble proteins. As yeasts are unable to use any peptides larger than a tripeptide (and the latter only slowly, Patterson and Ingledew, 1999; Ingledew and Patterson, 1999), there will always be residual FAN in the backset, but there is little *usable* FAN! This is the basis of the comment made previously that the quality of FAN (and thus the quality of backset) is of less value than many in the industry would believe.

The glucose content of these backset samples is also interesting. It can be concluded that one wheat plant was having considerable problems in fermentation at the time of the backset provision. Over 3% glucose was found in this backset; and this plant would have been suffering a loss of ethanol yield of about 15 g/L, a darker DDG after drying, and a reduced level of nitrogen in reused backset caused by Maillard reaction complexing of sugars with nitrogen sources during heating of the subsequent mash in the jet cooker.

As noted above, the concentrations of chemical components in thin stillage will increase as the backset recycles through successive fermentations. This was well recorded by Chin and Ingledew (1993; 1994) where a gradual increase in lactic acid to 1.4% was seen in infected mashes and where losses in yeast viability of up to 60% were shown. This occurred late in fermentation after ethanol was totally made to the rather low alcohol levels of 7-7.5% v/v predetermined by the mash used in Chin's work.

The impact of backset on the fermentation is because it contains any bacterial end products that are not removed in distillation (lactic acid and glycerol, for example), ions that were not taken up by the yeast in the previous fermentation(s), unused solubles from the original mash, insolubles from previous mashes, if any, and small amounts of volatiles which were not completely distilled. As the backset repeatedly recycles, the concentrations of some of these materials increase and some can become inhibitory.

Evaporator condensate water

Evaporators have been extensively used in the food industry in order to remove water from a fluid to obtain a more stable or reduced volume product, which is often later dried using other equipment. The condensed water from this process is hot, and can contain small amounts of the product, which during evaporation are distilled over in the concentrate as tiny particles in the mist. Therefore, evaporator concentrate is only pure in relative terms. It contains small amounts of a number of organic materials that are able to support microbial growth if the environment is conducive for the microbes bound to be present. In the food industry, the water can be recovered by reverse osmosis (purification of a water stream by forcing it under pressure through a membrane not permeable to the impurities it contains), providing savings in energy, water purchase, water processing on disposal, and softened water suitable for plant use. In fuel alcohol plants, the evaporator condensate is not processed by reverse osmosis. It is instead recycled to makeup water (without further processing) at the mash 'mingler', with the idea that a heat process in gelatinization/ liquefaction will sterilize the mash and kill any microorganisms. Little thought is given to the fact that microbial end products may already be present, and that they will survive the up-front processing of substrate. On arrival at the fermentor, they can be antagonistic to the yeast. Evaporator condensate components (those measured in the plants that collect data) are found in Table 9.

Table 9. Analytical data on evaporator condensate.

*Component**	*g/100 mL*
Ethanol	ND**
Methanol (or compound with same mobility)***	0.169
Sugars	
DP^{3+} (MW of maltotriose and larger)	0.017
DP^2 (MW of maltose)	0.013
DP^1 (MW of glucose)	0.065
Acids	
Lactic	ND
Acetic	0.037
Succinic	ND
Glycerol	0.015

*2 samples analyzed.

**Not detected. The heat of the process strips the ethanol if any remained. Note that other components present may not have been detected due to use of the routine fermentation column (Aminex HPX-87H).

***May in part or in whole be 2, 3 butanediol.

Carbon dioxide scrubber water

Carbon dioxide exiting fermentation is scrubbed by counter-current flowing water that removes ethanol and other compounds as well as any particulate materials entrained in the gas. The water is recycled - normally to slurry the corn – and is a substantial part of water input. This recycled wastewater stream can contain significant ethanol (Table 10) which, if heated too high in the mingler or slurry tank, would be lost to the alcohol plant. Some is lost in liquefaction, but an estimated 80% of the ethanol remains with the slurry during cooking and flashes off (to distillation towers) with the water vapors, or, in some plants, at the cooling tower. Some of the components identified in carbon dioxide scrubber water are shown in Table 10.

Side stripper bottom water

Side strippers handle the rectifier bottoms, and are engineered to strip the last concentrations of ethanol away from insolubles and less volatile residues exiting the rectifier. If the side stripper is engineered with direct steam injections (rather than as a reboiler), the side stripper bottoms will contain small amounts of organic residues. Table 11 shows the composition of compounds so far identified in side stripper bottoms. This water also shows the presence of a number of components that have been entrained in the water processed through the stills. The amounts are not large. It should be noted that the assessment of this sample has been done to date only with the Aminex column and refractive index HPLC (system commonly used for fermentation monitoring). The possibility for other compounds like fusel alcohols, and other organics not measured or identified by this column is not ruled out.

Table 10. Components of CO_2 scrubber water.

*Component**	*g/100 mL*
Ethanol	2.52
Methanol (or compound with same mobility)	0.115
Sugars	
DP^{3+} (MW of maltotriose and larger)	0.044
DP^2 (MW of maltose)	0.011
DP^1 (MW of glucose)	0.041
Acids	
Lactic	ND**
Acetic	ND**
Succinic	ND**
Glycerol	0.006

*5 samples analyzed
**Not detected

Table 11. Components of side stripper bottoms.

*Component**	*g/100 mL*
Ethanol	0.132
Methanol (or compound with same mobility)	ND**
Sugars	
DP^{3+} (MW of maltotriose and larger)	0.055
DP^2 (MW of maltose)	0.022
DP^1 (MW of glucose)	0.040
Acids	
Lactic	0.013
Acetic	0.019
Succinic	0.002
Glycerol	0.035

*22 samples analyzed
**Not detected

Interestingly, side stripper water also has been found to contain mineral content ranging from 9 ppm sodium (Na) to over 20 ppm phosphorus (P) and sulfur (S). Other ions including calcium (Ca), iron (Fe) and magnesium (mg) were all below 0.016 ppm. Extensive analyses have not been available to this author.

Implications of wastewater on yeast fermentation

Consideration of the inhibitory compounds found in the above recycled waters led us to considerable research and a diagram of the stresses that yeasts must endure. We now know that many of the stress factors act in a synergistic manner when more than one agent is found at inhibitory concentrations (Narendranath *et al.,* 2001). We also know that the presence of solids in the medium and the medium buffering capacity both have an influence on the inhibition of the growth of yeast by organic acids (Thomas *et al.,* 2002), as does the temperature of the fermention. Figure 2 (Ingledew, 1999) shows that a number of possible stress agents find their way into fuel alcohol fermentors via backset and by recycling of other wastewater streams within the plant. When concentrations increase to levels near or above the levels indicated (as single inhibitors), the yeast will not grow or metabolize at the same rate as is possible under ideal fermentation conditions (Thomas *et al*., 2001). In some cases, the levels of compounds such as acetic acid, lactic acid and ions like sodium are so high in fermentation that normal ethanol production is retarded. The immediate consequence in both continuous and batch fermentation is that unused sugars are sent to the beer well. This ensures that lower ethanol yields than those expected under ideal conditions are seen.

The concentrations of stress agents listed in Figure 2 have, for the most part, been determined as accurately as possible. Unfortunately, when medium conditions (mash type and concentration, pH, presence of additional stresses) change, so will the level of each 'inhibitor' examined that affects yeasts. We now know that the composition of the medium (i.e. corn mash) and the environmental conditions imposed (pH, temperature etc.) will influence the concentrations of any one inhibitor, which can affect yeast growth and metabolism. In fact,

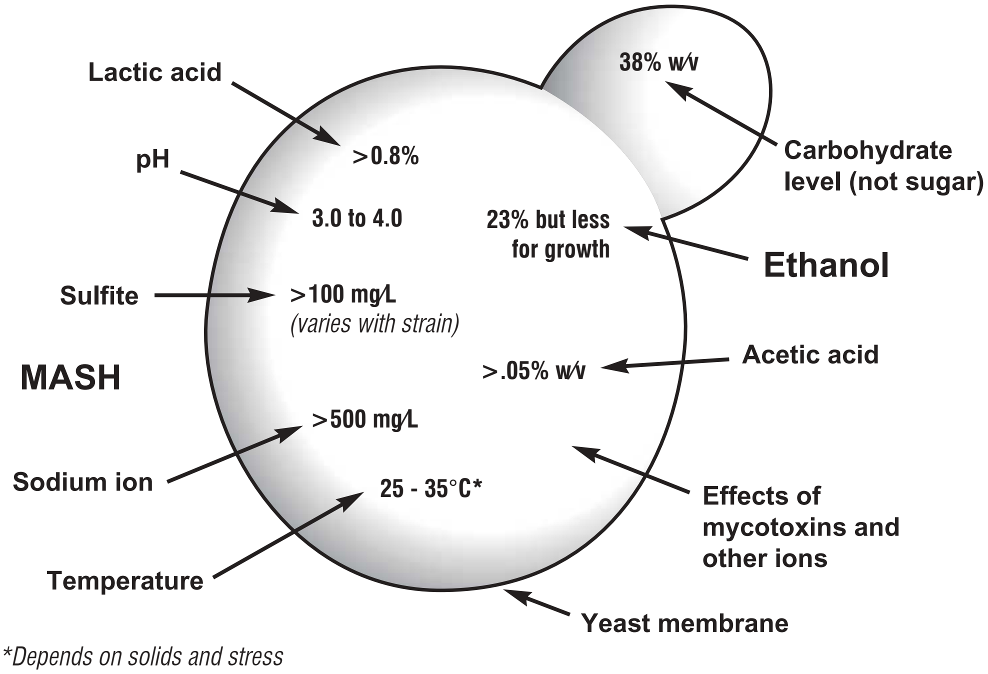


Figure 2. Stress factors that influence growth and metabolism of yeasts. Much of this work was done with a synthetic fermentation medium and may represent the smallest values of a single stress factor that is inhibitory to yeast.

inhibition by lactic acid and acetic acid is pH dependent (Narendranath, unpublished; Thomas *et al.,* 2002). As the knowledge of synergistic effects among inhibiting compounds and the magnitude of the effect of concentration all improve with further research, this stress diagram will be amended. The values shown are those we believe at present to be the minimum levels where effects on yeast can be measured.

Biomethanators

A more recent addition to the technology of fuel alcohol production is the anaerobic biomethanator. A process flow diagram of the ICM/Phoenix wastewater treatment system is reproduced in Figure 3 (Anon., 2003).

A biomethanator, in part, is a special type of fermentor, designed in this case to reduce by 90% the variable BOD/COD levels in evaporator condensate and other wastewaters. It produces methane and CO_2, the former of which is used to reduce boiler or dryer operation costs. Sulfur can also be removed from treated wastewaters by conversion to hydrogen sulfide. This is in some respects an exciting innovation with great potential to the industry: recycling treated water back to fermentors.

The anaerobic bioreactor in the biomethanator must be 'fed' a mix of nutrients needed to continue life of the bacteria on which the biomethanator is based – providing the food and environmental conditions that the bacterial population in the sludge require to grow. In addition, pH is adjusted with tank wash waters rich in sodium ions (used as NaOH for tank and line cleaning). Much of this sodium ion is converted to sodium carbonate (Na_2CO_3) by the carbon dioxide in the system. Regardless, the sodium ion, all unused nutrients, and all end products produced from the biomethanator that were not converted to methane and carbon dioxide move through the biomethanator unabated. This results in high levels of sodium ion and perhaps a series of yet-to-be-identified compounds in subsequent fermentors. It is uncertain, as yet, whether the levels of sodium experienced in plants both with and without methanators are high enough to inhibit yeasts. Evidence accumulated in this lab, however, using corn mash similar to that made in dry mills, would indicate that large amounts of sodium ion would lead to a purging requirement to eliminate

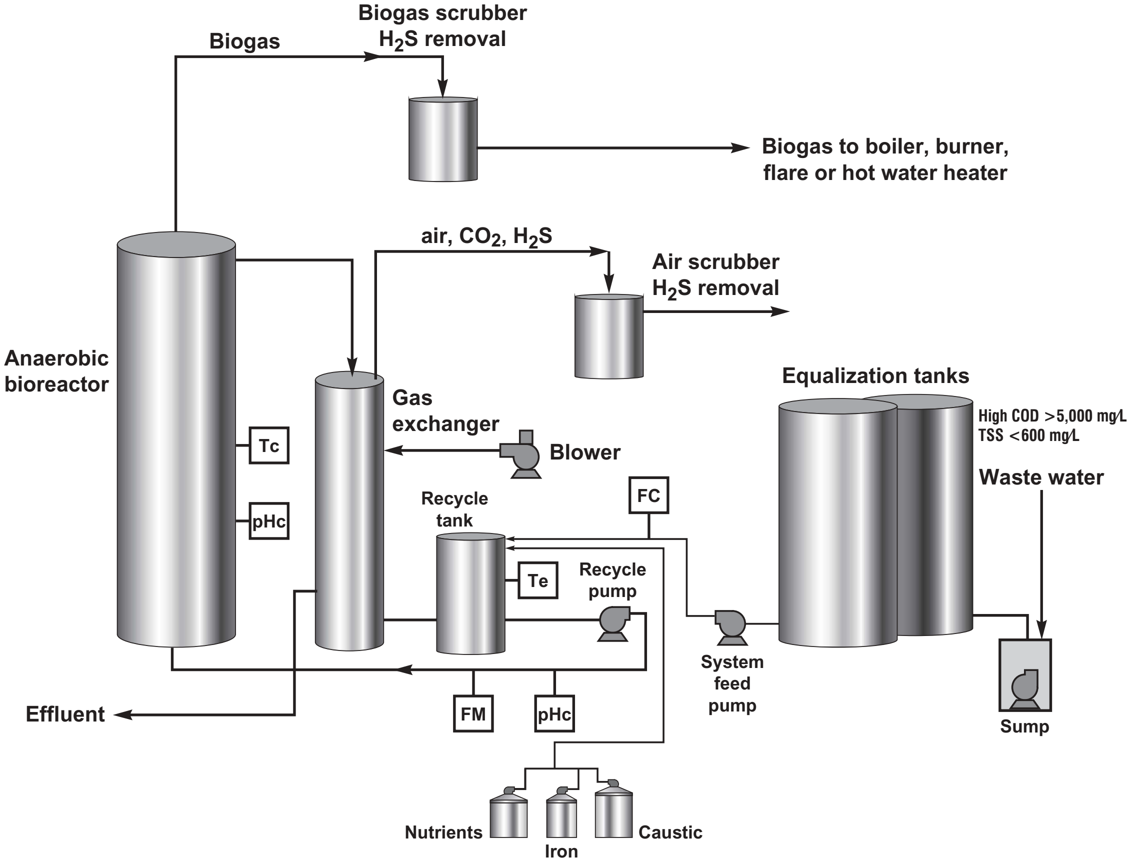

Biogas
Biogas scrubber
H_2S removal
Biogas to boiler, burner, flare or hot water heater
air, CO_2, H_2S
Air scrubber
H_2S removal
Anaerobic bioreactor
Gas exchanger
Blower
Equalization tanks
High COD >5,000 mg/L
TSS <600 mg/L
Waste water
Tc
pHc
Recycle tank
Te
Recycle pump
FC
System feed pump
Sump
Effluent
FM
pHc
Nutrients
Iron
Caustic

Figure 3. The ICM/Phoenix biomethanator.

ion buildup in water that would otherwise reenter the plant via the methanator.

The levels of sodium needed to inhibit laboratory fermentation of corn mash when no other stressful agents are present would appear to be between 2500 and 5000 ppm (Figure 4). This should allow plenty of leeway for the use of methanator effluent in fermentation (at least from the sodium standpoint). It should not be forgotten, however, that when more than one stressful agent is present, the effects are synergistic, and at this point, much lower concentrations of sodium (near 500–600 ppm) have been seen to have an effect on the yeast.

When biomethanators are not used, it is common practice to use a combination of evaporator condensate water, carbon dioxide scrubber water, side stripper bottoms water, and well water for the cooking process. Note that sodium hydroxide washwater is also unevenly recycled to fermentors in volumes averaging about 0.5% of plant flow rate. It is this volume of waste sodium ion that is potentially dangerous if added to the cook or directly to fermentors. The disposal of sodium ion even through the dryers via incorporation into DDG may require assessment.

Anecdotal evidence received from production plants in the past (prior to methanator technology) indicated that levels of sodium ion above 500 mg/L would inhibit yeast growth and fermentation; and this was published in the stress diagram (Figure 2) found in the last edition of *The Alcohol Textbook* (Ingledew, 1999). In the past, evidence published by Maiorella *et al.* (1984) showed that a sodium concentration of ~5500 mg/L sodium was required to demonstrate negative effects on yeast as manifested by >20% inhibition. Moreover, Watson (1970) showed that in batch culture, 0.25 to 1.5 M NaCl (5750 to 33700 ppm Na) was needed to affect growth rate and the yield of cells grown on glucose. Jones and Greenfield (1984) stated that for most tolerant strains of yeast, total inhibition of growth

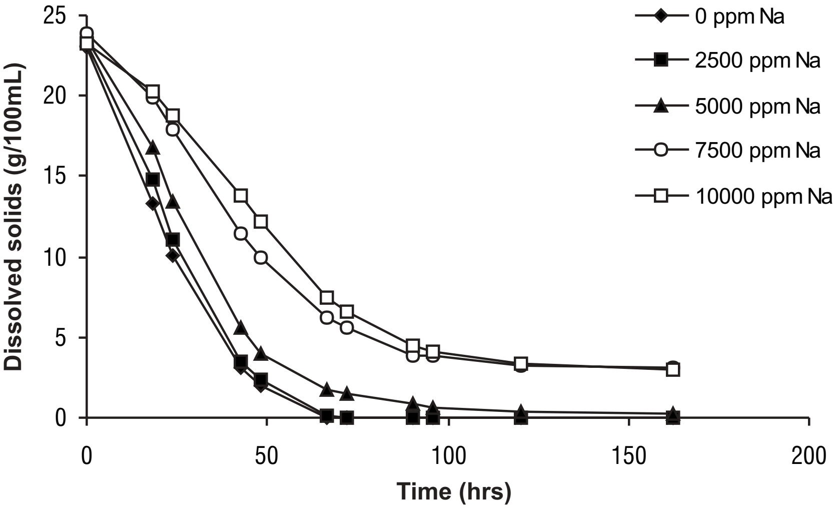


Figure 4. The effect of sodium ion as a single stress factor on the utilization of sugar during fuel alcohol production (Hynes *et al.*, 2003, unpublished data).

occurs above 1-2 M (23,000-46,000 ppm Na), the level needed for non-sensitive yeasts. For sensitive strains, 0.1 M or 2300 ppm was needed. These latter values are in similar range to Figure 4. Insensitivity to sodium is said to be via the diversion of energy needed to maintain an electrochemical gradient of sodium ion against passive or facilitated diffusion into the cells. The effect was not the same with different yeasts measured. Explanation of the discrepancy between the numbers that would definitely be inhibitory and literature values may be found in the work of Jones and Gadd (1990), who indicated that the ratio between sodium and potassium is very important; and that at a pH of 5.0 when the Na:K ratio is about 20, potassium uptake is inhibited by 70%. At pH 4, sodium uptake becomes negligible; so they indicated that in an industrial medium containing high levels of sodium, a pH around 4.0 would be required to overcome the deleterious effects of the sodium ion. The optimal concentrations of K^+ ion of 2-4 mM may be in part due to the presence of sodium in mash.

The differences seen in an industrial plant between organic acid problems (caused by bacteria and recycle of stillage) and ionic problems (probably caused by recycle of NaOH wash waters by stillage and biomethanator action) are shown in Table 12 in comparison to control fermentations where inhibitory levels of neither type of inhibitor were present.

Table 12. Plant data demonstrating the effects of both organic acids and sodium ion on batch fermentations.

Tank no.	*Ethanol (% w/v)*	*Glucose (% w/v)*	*Lactic acid (% w/v)*	*Acetic acid (% w/v)*	*Sodium (ppm)*	*Ethanol loss (%)*
A	12.4	0.03	0.35	0.02	<290	-
B	12.8	0.24	0.29	0.04	<290	-
H	10.8	4.70	1.0	0.32	<290	14.3
K	9.5	5.14	1.0	0.41	<290	26.2
M	11.5	1.40	0.25	0.0	660	8.7
N	10.8	1.29	0.17	0.01	720	14.3

It can be seen from Table 12 that under normal operational conditions (A and B), this alcohol plant was capable of making over 12% alcohol under conditions where lactic and acetic acids were low and sodium was not present at alarming levels. When organic acids were present at inhibiting levels (H and K), ethanol yields dropped, sugar ‘bleed’ at end fermentation was high, and the acids circulated into other fermentors in backset. This evidence strongly supports the synergistic or at least concerted inhibitory effects of lactic and acetic acids. Under conditions of high sodium in Tanks M and N, where the source of sodium was from tank washings recirculated with backset, ethanol levels fell by 1-2 g/100 mL, and glucose levels rose accordingly. Sodium levels of >500 mg/L therefore appeared to significantly lower ethanol output of this plant. The magnitude of ethanol loss in these examples demonstrates the problems a plant can experience when there is no opportunity to remove recycled thin stillage from the system in order to lower the levels of recycled chemicals and preserve the delicate biological conditions in the fermentor. It is the author's opinion that methanators can lead to such elevated levels of ions (but not organic acids) unless there are undetermined toxic bioproducts of methane-producing microbes that find their way back into the fermentation system. This is as difficult a situation to monitor and rectify, as is the present case with continual recycle of backset.

Conclusions: the importance of elimination or preventing buildup of inhibiting chemicals

It has been estimated that the fuel alcohol industry in general loses from 1 to 4% of potential alcohol volume each year. The North American industry produces at this writing almost three billion gallons per year. A 1% loss is 30 million gallons (113 million liters), with a value of approximately $33 million. A 1% loss in a plant such as the one in Table 12 would only be 0.12% alcohol. Such a loss is unlikely to be noticed due to variability in chemical quality control assays and the starch levels in incoming substrate. A 4% loss over the whole North American industry is 120 million gallons (452 million liters) with an approximate value of $132 million. In certain plants (sometimes under particular but not infrequent circumstances as seen in Table 12), losses greatly exceed these percentages. A major cause of such losses is bacterial degradation of sugars that should have been converted by yeast to ethanol. All efforts should therefore be made in plants to avoid bacterial action. The action of bacteria or wild yeasts, or the effects of their end products of metabolism, which remain after heat or other agents kill the microbes that made them, are significant causes of yield loss. One must not forget, however, that chemicals outside of those made by microbes can also affect the process – sodium ions from tank and line washings are a case in point.

The message for the ethanol industry is that 'zero effluent' plants (those which do not release significant volumes of water with high BOD or COD or high concentrations of salts) are not easily designed – especially when the prime mode of water recycle is via the fermentation system. Fermentors (biologically fragile reactor systems) are susceptible to all chemicals added to them. Engineering to save on water usage is contrary to the preservation of the physiological conditions required by the yeast to ensure complete and rapid fermentation.

References

Anonymous, 2003. The ICM/Phoenix biomethanator. *http://www.phiosystems.com/experience.htm*.

Bryan, T. 2003. Green Machines. Ethanol Producer 9(7):12-15, 52, 55-56.

Chin, P.M. and W.M. Ingledew. 1993. Effect of recycled laboratory backset on fermentation of wheat mashes. J. Agric. Food Chem. 41:1158-1163.

Chin, P.M. and W.M. Ingledew. 1994. Effect of lactic acid bacteria on wheat mash fermentations prepared with laboratory backset. Enz. Microb. Technol. 16:311-317.

Ingledew. W.M. 1993. Yeasts for production of fuel ethanol. In: *The Yeasts* Vol. 5. 2nd Edition. Academic Press, N.Y. pp 245-291.

Ingledew, W.M. 1995. Chapter 7. The biochemistry of alcohol production. In: *The Alcohol Textbook.* Second Edition. (T.P. Lyons, D.R. Kelsall, and J.E. Murtagh, eds). Nottingham University Press. Nottingham, UK. pp. 55-79.

Ingledew, W.M. 1999. Chapter 5. Alcohol Production by *Saccharomyces cerevisiae*: a yeast primer. In: *The Alcohol Textbook. Third Edition.*(K. Jacques, T.P. Lyons and D.R. Kelsall, eds). Nottingham University Press. Nottingham, UK. pp. 49-87.

Ingledew, W.M. and C.A. Patterson. 1999. Effect of nitrogen source and concentration on the uptake of peptides by a lager yeast in continuous culture. J. Amer. Soc. Brew. Chem. 57:9-17.

Jones, R.P. and G.M. Gadd. 1990. Ionic nutrition of yeast – physiological mechanisms involved and implications for biotechnology. Enz. Microb. Technol. 12(6):402-418.

Jones, R.P. and P.F. Greenfield. 1984. A review of yeast ionic nutrition. Part 1: Growth and fermentation requirements. Proc. Biochem. 19(2):48-60.

Jones A. and W.M. Ingledew. 1994. Fermentation of very high gravity wheat mash prepared using fresh yeast autolysate. Bioresource Technol. 50:97-101.

Maoirella B.L., H.W. Blanch and C.R. Wilke. 1984. Feed component inhibition in ethanol fermentation by *Saccharomyces cerevisiae*. Biotech Bioeng 26:1155-1166.

Narendranath, N.V., S. H. Hynes, K.C. Thomas and W.M. Ingledew. 1997. Effects of lactobacilli on yeast-catalyzed ethanol fermentations. Appl. Environ. Microbiol. 63: 4158-4163.

Narendranath, N.V., K.C. Thomas and W.M. Ingledew. 2001a. Effects of acetic acid and lactic acid on the growth of *Saccharomyces cerevisiae* in a minimal medium. J. Indust. Microbiol. Biotechnol. 26:171-177.

Narendranath, N.V., K.C. Thomas and W.M. Ingledew. 2001b. Acetic acid and lactic acid inhibition of growth of *Saccharomyces cerevisiae* by different mechanisms. J. Amer. Soc. Brew. Chem. 59:187-194.

Patterson, C.A. and W.M. Ingledew. 1999. Utilization of peptides by a lager brewing yeast. J. Amer. Soc. Brew. Chem. 57:1-8.

Thomas, K.C., S.H. Hynes and W.M. Ingledew. 2001. Effect of lactobacilli on yeast growth, viability and batch and semi-continuous alcoholic fermentation of corn mash. Journal of Applied Microbiology. 90:819-828.

Thomas, K.C., S.H. Hynes and W.M. Ingledew. 2002. Influence of medium buffering capacity on inhibition of *Saccharomyces cerevisiae* growth by acetic and lactic acids. Applied and Environmental Microbiology 68:1616-1623.

Watson, T.G. 1970. Effects of sodium chloride on steady-state growth and metabolism of *Saccharomyces cerevisiae*. J. Gen. Microbiol. 64:91-99.

Chapter 25

Understanding energy use and energy users in contemporary ethanol plants

JOHN MEREDITH

President, Ro-Tech, Inc., Louisville, Kentucky, USA

Results of calculations quantifying the thermal and electrical energy needed to convert starch and extract ethanol from grain are often controversial and depend on the assumptions behind the calculations. Looking through the literature and surveying historical operating costs for beverage distilleries and ethanol plants reveals a wide range of results reported for energy use per gallon of ethanol produced. It is also noteworthy that total energy use per gallon of ethanol produced in beverage plants in the late 1900s was significantly greater than the energy use per gallon in today's new fuel ethanol plants.

Terms defined and assumptions for energy use calculations

For purposes of this chapter, the criteria below will apply to the calculations and conclusions which follow:

Gallon of denatured ethanol. One US gallon of ethanol containing 4.76% denaturant and in agreement with ASTM D4806.

Thermal energy. Based on natural gas used at 1000 BTU/ft^3 at standard conditions (60°F, 1 atm of pressure).

Electrical energy. Kilowatt hours used by the plant. Demand and energy cost components of typical utility electrical bills are not considered in this analysis. These two components are measured by utility companies and used to determine monthly electrical bills. This cost is also a function of a number of factors outside the scope of this chapter (plant location, energy market at any particular time, etc.).

Feedstock. Energy use in ethanol plants is dependent on feedstock. For our purposes, feedstock will be No. 2 corn at approximately 15% moisture. Some ethanol plants have experimented with using 'wet corn' at ~30% moisture as a feedstock. This technique would eliminate the energy need for drying the corn to 15% moisture at the elevator. However to date, these efforts have been mostly unsuccessful due to problems in handling and preparing the corn for cooking.

Other feedstocks require similar energy input to produce ethanol with some variation. Wheat is virtually identical to corn in energy requirements except as affected by moisture content. Rye and milo (sorghum) require some additional energy due to higher slurry viscosities, which affect heat transfer and pumping. Sugar cane-based feedstocks use less energy (no dryhouse) compared to grain-based feedstock plants with DDGS facilities.

Boundaries. Energy use for purposes of this chapter starts at grain unloading at the ethanol plant and finishes with product at the point ready to be pumped to a waiting truck or rail car. Some studies have investigated energy use starting with corn in the field and finishing with ethanol at a receiving terminal separated from the plant. A recent study by the US Department of Agriculture stated that ethanol plants deliver 34% more energy to the end user of the fuel than is used in growing, harvesting and distilling corn into ethanol. Ethanol releases 90,030 BTU for each gallon burned. If 90,030 BTU represents a 34% increase, then the total energy required to make ethanol would be approximately 67,000 BTU. Modern plants require approximately 36,000 BTU of thermal energy and 1.1 KWH (3800 BTU/gallon) of electrical energy per gallon of denatured ethanol. This totals 39,800 BTU/gallon, which leaves over 25,000 BTU/gallon for energy users outside the plant boundaries including farm equipment involved in planting and harvest, energy to transport the corn from the field to an elevator for drying and then to the ethanol plant, energy needed to dry corn to ~15% moisture, and energy expended in transporting ethanol from plant to receiving terminal.

Despite the focus some place on the overall energy balance of ethanol production, it is worth remembering that ethanol is not added to fuel for its energy value. The Clean Air Act of 1990 required adding oxygenates to gasoline in some locations to reduce emissions. Ethanol is an oxygenate, and is compared in Table 1 to other oxygenates. Methyl tertiary butyl ether (MTBE), the oxygenate of choice until a few years ago, has been found to be a pollutant, which has led to increased interest in ethanol as a replacement.

Table 1. Comparison of four fuel oxygenates.

	Oxygen (%)
Ethanol	34.73
Methanol	49.90
MTBE (Methanol and isobutylene)	18.15
ETBE (Ethanol and isobutylene)	15.66

Energy use for co-product handling, drying and storage will be included. Although some older beverage plants show a net loss in the production of DDGS when comparing costs to revenues, drying co-products to a DDGS product is considered profitable in newly constructed, more energy efficient, ethanol plants. Energy use in the dryhouse is a major component of the plant energy balance.

Beverage plants versus fuel ethanol plants. While this chapter focuses on energy use in fuel ethanol plants, the relationships between energy use and plant system design apply equally to both industries. When comparing beverage plants to similarly designed fuel ethanol plants, beverage plants consume more energy per gallon owing to the specifics of producing a defined product for human consumption. Distillation is often more complicated in order to produce a product with controlled amounts of congeners. In addition, heat recapture techniques, thick slurries and hot water recycling are not as prevalent due also to quality concerns.

Understanding thermal and electrical energy usage ratios

BTU (THERMAL ENERGY) PER GALLON OF ETHANOL

Modern dry milling mid-sized (40 million gallons per year) ethanol plants are being constructed in the US based on an expected thermal energy requirement of 36,000 BTU (natural gas) per gallon of ethanol. This energy requirement is based on denatured ethanol. In other words, the ratio is calculated using a mix of 100 gallons of undenatured plant product with 5 gallons of denaturant (low octane gasoline) yielding 4.76% denaturant in the mix. Some plants look at the ratio before denaturing, which would be 36,000 BTU/gallon ethanol x 105 gallons denatured/100 gallons undenatured ethanol = 37,800 BTU/gallon. Likewise, other plants use ‘steam BTUs’ instead of ‘natural gas BTUs’. ‘Steam BTUs’ introduce boiler efficiency into the equation as follows:

For denatured ethanol:

$$\frac{\text{36,000 BTU (gas)}}{\text{gal ethanol}} \times \frac{\text{0.82 steam BTU}}{\text{1.0 natural gas BTU}} = \frac{\text{29,520 steam BTUs}}{\text{gal ethanol}}$$

For ethanol before denaturing:

$$\frac{\text{29,520 steam BTU}}{\text{gal undenatured ethanol}} \times \frac{\text{105 gal}}{\text{100 gal undenatured ethanol}} = \frac{\text{30,996 steam BTUs}}{\text{gal undenatured ethanol}}$$

ELECTRICAL ENERGY (KILOWATT HOURS) PER GALLON OF ETHANOL

Electrical use in a modern mid-sized plant is expected to be between 0.7 and 1.2 KWH/gallon ethanol. Using 1.1 KWH/gallon as a conservative average for denatured ethanol, and calculating the value when denaturing is removed from the equation, the following is obtained:

$$\frac{\text{1.1 KWH}}{\text{gal ethanol}} \times \frac{\text{105 gal denatured ethanol}}{\text{gal undenatured ethanol}} = \frac{\text{1.155 KWH}}{\text{gal undenatured ethanol}}$$

RELATIONSHIP BETWEEN THERMAL AND ELECTRICAL ENERGY USE

To further complicate thermal and electrical energy use statements, the usage ratios can be dramatically changed by plant design techniques that transfer energy sources from thermal to electrical (or electrical to thermal) for certain plant processes. For example, the entire dryhouse evaporator thermal energy load can be shifted to an electrical load by replacing a multiple effect steam driven evaporator with a mechanical vapor recompression type with electric driver.

Factors affecting energy use and efficiency

MASH DILUTION

Any extra water added to the corn meal slurry system before cooking must be heated three times in the ethanol plant. Without heat recycle and recapture, some of the extra water is evaporated twice, requiring over 16,000 BTU for each gallon of water as it moves through these processes:

- cooking (raising the fluid's condition from a saturated liquid at ambient temperature to a saturated liquid at approximately 230°F)
- distilling and purifying (changing fluid state from a saturated liquid (beer) at 90°F to a partly saturated vapor at 200 proof and the rest to a saturated liquid at approximately 220°F)
- dryhouse operation (changing conditions from a saturated liquid at 220°F (stillage) to a saturated vapor at 160°F to 230°F)

Modern ethanol plants reduce the water and backset added to the corn meal in the meal slurry tank to a level acceptable in terms of conversion, yield and general handling characteristics. There is, however, a tradeoff between concentrating the slurry to reduce evaporation costs and the subsequent increase in enzyme and chemical cost to maintain the proper fluid viscosity and pH needed for cooking and fermentation.

Keeping the mixture of corn meal and water in the slurry tank at a ratio of 1 lb corn meal: 2 lbs water + backset total provides a concentrated slurry that saves energy and leads to good conversion and alcohol yield. This 1:2 mixture will yield an ethanol concentration in the beer of approximately 14% abv. Diluting the mixture to 1:2.5 gives an ethanol concentration in the beer of approximately 12% abv. The plant running at 12% abv will use as much as 20% more steam in the cooking, distilling and dryhouse operation compared to the plant running at beer concentrations of 14% abv.

The new high temperature and alcohol resistant yeasts can operate at 95°F and 18% abv (or more) in a fermentor, further decreasing energy requirements. However, slurry handling problems and heat transfer problems increase with these thicker beers.

NON-ENERGY DEPENDENT YIELD ENHANCERS

Alcohol yield per bushel of corn is calculated and expressed as follows:

1000 kg corn at 71% starch = 710 kg starch.
At 100% efficient conversion 362.8 kg ethanol would be produced.
At 90% efficient conversion 326.5 kg (718.3 lb) ethanol would be produced.

= 108.8 gallons (6.6 lb/gallon)
= 108.8 gallons per 1000 kg corn
= 2.77 gallons per bushel (56 lbs) of corn

Typically ethanol plant yields are closer to 2.65 gallons/bushel after distillation and final processing. If alcohol yields per bushel of grain increase due to non-energy related procedures, then thermal and electrical energy ratios will improve. For example, Alltech's solid state enzyme Rhizozyme™ increases alcohol yield per bushel of grain by 10% or more versus the use of traditional enzymes. This 10% increase in yield per bushel of grain will have a directly proportional 10% impact on the thermal and electrical energy ratios before denaturing.

CAPITAL IMPROVEMENTS, EQUIPMENT SELECTION AND ENERGY USAGE RATIOS

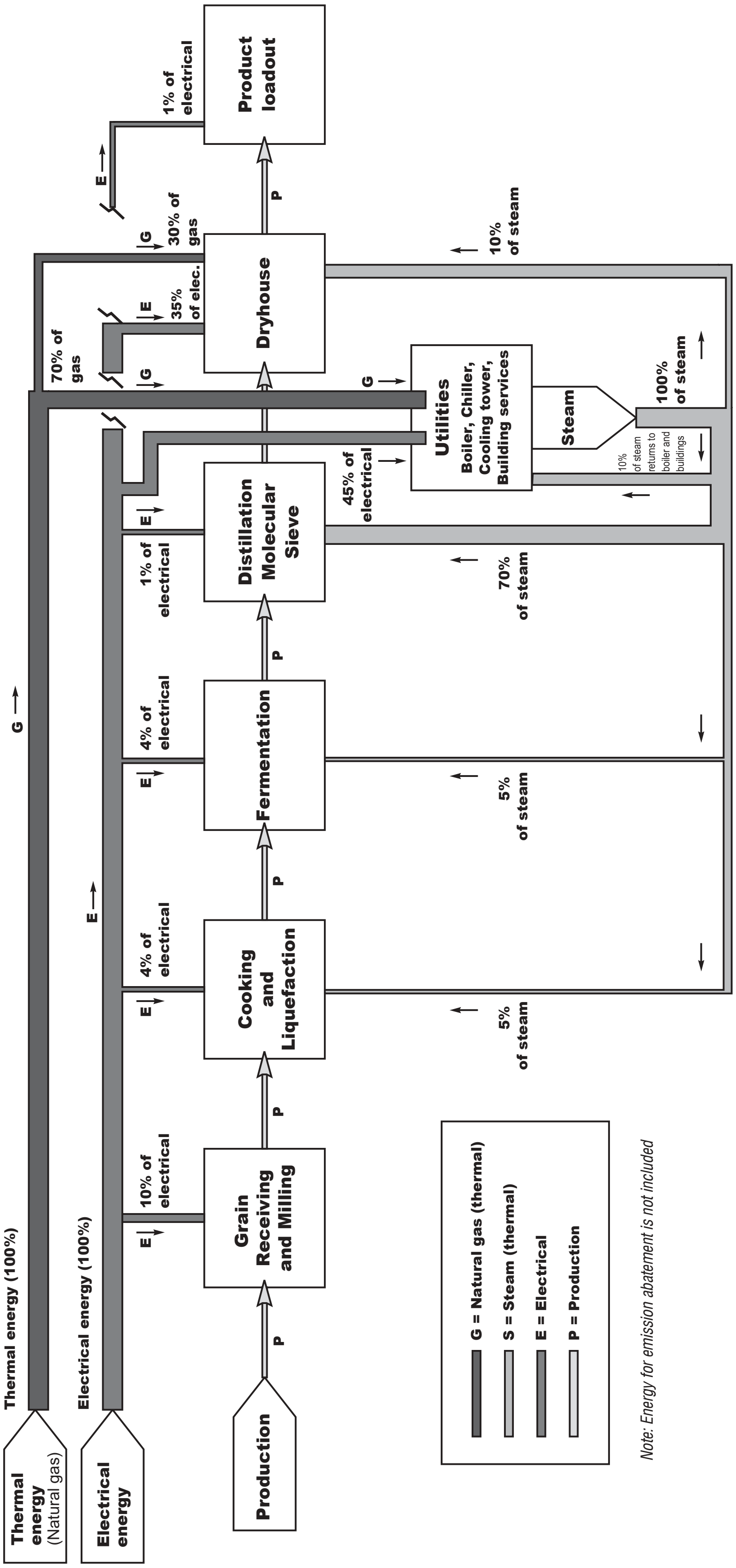
The ratios are dependent on capital improvements built into plant design that reduce energy use. Heat recovery, heat interchange, etc. will reduce energy use ratios. Elimination of the dryer by selling wet distillers grains with solubles (WDGS) instead of dry distillers grains with solubles (DDGS) will dramatically improve the thermal energy ratio. This may not, however, improve the plant profits depending on the relative sale price of WDGS versus DDGS.

HEAT RECLAMATION TECHNIQUES

Listed below are design features presently in use in ethanol plants. The process designer has a choice in selecting one or more of these features with return on investment (operating cost *vs* capital cost tradeoff) the governing factor.

- Preheat the water used in cooking or use recycled hot water:
    - use treated evaporator condensate
    - use rectifying column bottoms
    - use distillation column overhead condenser water
    - interchange with mash to be cooled after cooking
- Preheat the beer for distillation:
    - interchange with mash to be cooled after cooking
    - interchange with rectifying column bottoms
- Pre-heat or pre-evaporate thin stillage:
    - interchange with rectifying column overheads
    - interchange with molecular sieve overheads
    - interchange with evaporator condensate
- Recover the saturated liquid flash from the bottom of the stripping column and return back to stripping column
- For plants with thermal oxidizers on dryer vents
    - use regenerative type thermal oxidizer and provide time for clean up (bake out) in the operating cycle
    - for non-regenerative types, use waste heat boiler on oxidizer discharge and produce steam for the plant
- Interchange molecular sieve inlet and outlet streams
- Use steam turbine drives and use resulting low pressure steam for distillation and dryhouse systems

Maximizing use of these techniques allows some ethanol plants to approach the 36,000 BTU/gallon and 0.7 KWH/gallon ratios noted above. Without these energy saving improvements, thermal energy use will increase dramatically with ratios of 75,000 BTU/gallon and higher a possibility.


Thermal energy (Natural gas)
Electrical energy
Production
Thermal energy (100%)
Electrical energy (100%)
G
E
P
Grain Receiving and Milling
Cooking and Liquefaction
Fermentation
Distillation Molecular Sieve
Dryhouse
Product loadout
Utilities Boiler, Chiller, Cooling tower, Building services
Steam
10% of electrical
4% of electrical
4% of electrical
1% of electrical
45% of electrical
35% of elec.
1% of electrical
70% of gas
30% of gas
5% of steam
5% of steam
70% of steam
10% of steam
100% of steam
10% of steam returns to boiler and buildings
90% of steam
10% of steam
G = Natural gas (thermal)
S = Steam (thermal)
E = Electrical
P = Production

Note: Energy for emission abatement is not included

Figure 1. Energy flow through an ethanol plant.

Distribution of energy use in an ethanol plant

Table 2 details energy use by subsystem in an ethanol plant. An overview is depicted in Figure 1. Energy use distribution in a beverage plant is similar. Data in Table 3 were taken from a new grassroots beverage plant design in 1990.

Table 2. Ethanol plant energy use by subsystem[1,2].

	Thermal (% of total)	*Electrical (% of total)*
Grain receiving and milling	Negligible	10-15
Cooking and liquefaction	5-8	1-5
Fermentation	1	1-5
Distillation/molecular sieve	55-60	1-5
Product loadout	Negligible	1-5
Evaporation and drying (including emissions)	40-45	30-40
Utilities	5-7	40-50
Buildings	1	1

[1] Based on data collected by Ro-Tech from plants constructed since 1995.

[2] Emission abatement no included.

Table 3. Beverage plant energy use by subsystem.

	Thermal (% of total)	*Electrical (% of total)*
Grain receiving and milling	Negligible	16
Cooking and liquefaction	7.4	5
Fermentation	1.0	1
Distillation and product handling	32.1	2
Evaporation and drying (including emissions)	53.5	41
Utilities	5	34
Buildings	1	1

The primary thermal energy use points are distillation/purification and the dryhouse followed by the cooking process. Cooking is a direct live steam injection process using a combination of heat, time and enzymes to liquefy the starch. Indirect heat transfer systems have been tried with little success. Distillation systems in ethanol plants include stripping and rectifying columns (sometimes combined into one column) yielding alcohol at a concentration approaching the azeotrope of 95.6% ethanol by weight. In most beverage plants, these columns are heated by live steam while fuel ethanol plants use reboilers. Reboilers eliminate beer dilution associated with live steam and also permit recapture of clean condensate heat by the plant's boiler system.

Purifying the alcohol water mixture after distillation from approximately 192 proof to 200 proof is accomplished in most ethanol plants using molecular sieve technology, which requires thermal energy to vaporize the feed to the sieve and then more energy to superheat the vapor to over 300°F before entering the sieve. Some energy can be saved by sending rectifying column overhead vapor direct to the sieve superheater without first condensing the rectifying column vapor. However, combining the continuous process rectifier with the batch process sieves can present control problems for the ethanol plant operator.

Dryhouses use thermal energy to boil and/or dry the stillage, removing condensed water in the evaporator and vapor from the dryer stack. Depending on equipment selection, various combinations of steam, natural gas, propane or fuel oil are used to provide the thermal energy. Large electrical energy use points (large motors) in an ethanol plant are concentrated in utility areas (chillers, air compressors, cooling towers, boiler fans). If the evaporator is a mechanical vapor recompression system, then the dryhouse becomes a major electrical user.

The grain handling and milling areas will use significant amounts of power depending on use of pneumatic systems as well as the complexity of the grain receiving, storage, transfer and milling systems. However, whereas the modern fuel ethanol plant approaches energy usage ratios of 36,000 BTU/gallon and 0.7 KWH/gallon, the 1990 beverage plant in Table 3 had a similar electrical ratio but the thermal ratio was 73,000 BTU/gallon (calculated as one gallon of 200 proof ethanol).

Overall ethanol plant energy balance

Energy inputs and energy exits for an ethanol plant are listed in Table 4. Chemical and physical properties (including temperature, pressure and specific heat) of each item must be known to calculate the proper enthalpy (BTU/lb) associated with entering or exiting energy.

Table 4. Energy inputs and energy exits for an ethanol plant.

Energy entering	*Energy leaving*
Corn	Denatured ethanol, including heads and fusel oils
Make up water	Carbon dioxide
Fuel	Wastewater emissions to land application, outfall (stream), or sewer (from make up water treatment, from process (evaporator, CIP, other), from boiler, scrubbers and cooling tower blowdowns)
Electricity	
Denaturant	Air emissions and waste heat to atmosphere (from dryer or oxidizer, tank and vessel vents, cooling towers, baghouses, from thermal losses through exterior vessel walls and building walls, from exhaust air (buildings)
Yeast	
Enzymes	Waste solids
Make up air (boiler, dryer, pneumatic systems, building space)	Wastewater sludge, CIP screenings, corn screenings (‘overs’)
	Co-products
Chemicals (for CIP, fermentation nutrients)	

Conclusion

Modern ethanol plant designers are faced with a variety of choices when selecting process systems and process equipment. After the systems have been specified and selected, energy usage ratios can be projected. Conservative beverage plant designs have a long track record of dependable operation but often pay an operating cost penalty with thermal ratios approaching 75,000 BTU/gallon. Aggressive energy-reducing designs for modern fuel ethanol plants produce ratios approaching 36,000 BTU/gallon of ethanol and 0.7 KWH/ gallon of ethanol. However, the cost of capital improvements to reach these numbers must be justified; and the reliability of the resulting capital equipment must be proven.

The dryhouse, co-products and the future

Chapter 26

Dryhouse design: focusing on reliability and return on investment

JOHN MEREDITH

President, Ro-Tech, Inc., Louisville, Kentucky, USA

Introduction: an overview of distillery co-products

Production of ethyl alcohol by fermentation of naturally occurring starch or sugar-containing agricultural products is a well-established and complex industry today. A variety of feedstocks are utilized, however corn, wheat, rye, milo (sorghum) and sugar cane are the major sources of fermentable sugar.

Depending on the industry and application, co-products from these feedstocks include: distillers dried grain with solubles, distillers dried grain without solubles, distillers solubles, wet distillers grains with solubles, corn oil, yeast cells, thick wet stillage, thin wet stillage, centrifuge cake, evaporator syrup, corn gluten meal, corn gluten feed, starch, sugar, carbon dioxide, and fusel oils.

Two processes based on corn as a feedstock have evolved over the last few hundred years. Wet milling, dating back to the mid 1800s, requires wet steeping of the corn kernel before processing followed by a series of unit operations aimed primarily at starch recovery. Dry milling, which dates back hundreds of years, requires grinding or 'milling' of the corn kernel into a flour before further processing. Processes generating co-products from the dry mill will be the focus of this chapter.

After fermentation in a dry mill corn feedstock plant, ethyl alcohol is recovered by distillation. Depending on the ethyl alcohol product type (fuel grade ethanol, beverage spirit including neutral spirits, bourbon, gin, etc.), distillation systems and equipment vary. However in all cases, the spent residue from the distillation process, commonly called whole stillage, is the source of the dry mill co-products noted in Figure 1. Whole stillage normally passes over a screen or through a liquid/solid separation device and the thin cut (backset) is returned to fermentation. The remainder is collected as either thick stillage or additional thin stillage and cake.

Chemical and physical properties of whole stillage are determined by the ethanol production process employed. The feedstock, mash formulation, set fermentor constituents and distillation system design all are important in determining the nature of distillery co-products. Quantities of stillage available for recovery are also determined by process considerations including the grain concentration in the fermentor and the amount of backset recycled to fermentation.

Most dry mill plants in the early 1900s and before were small corn milling operations with

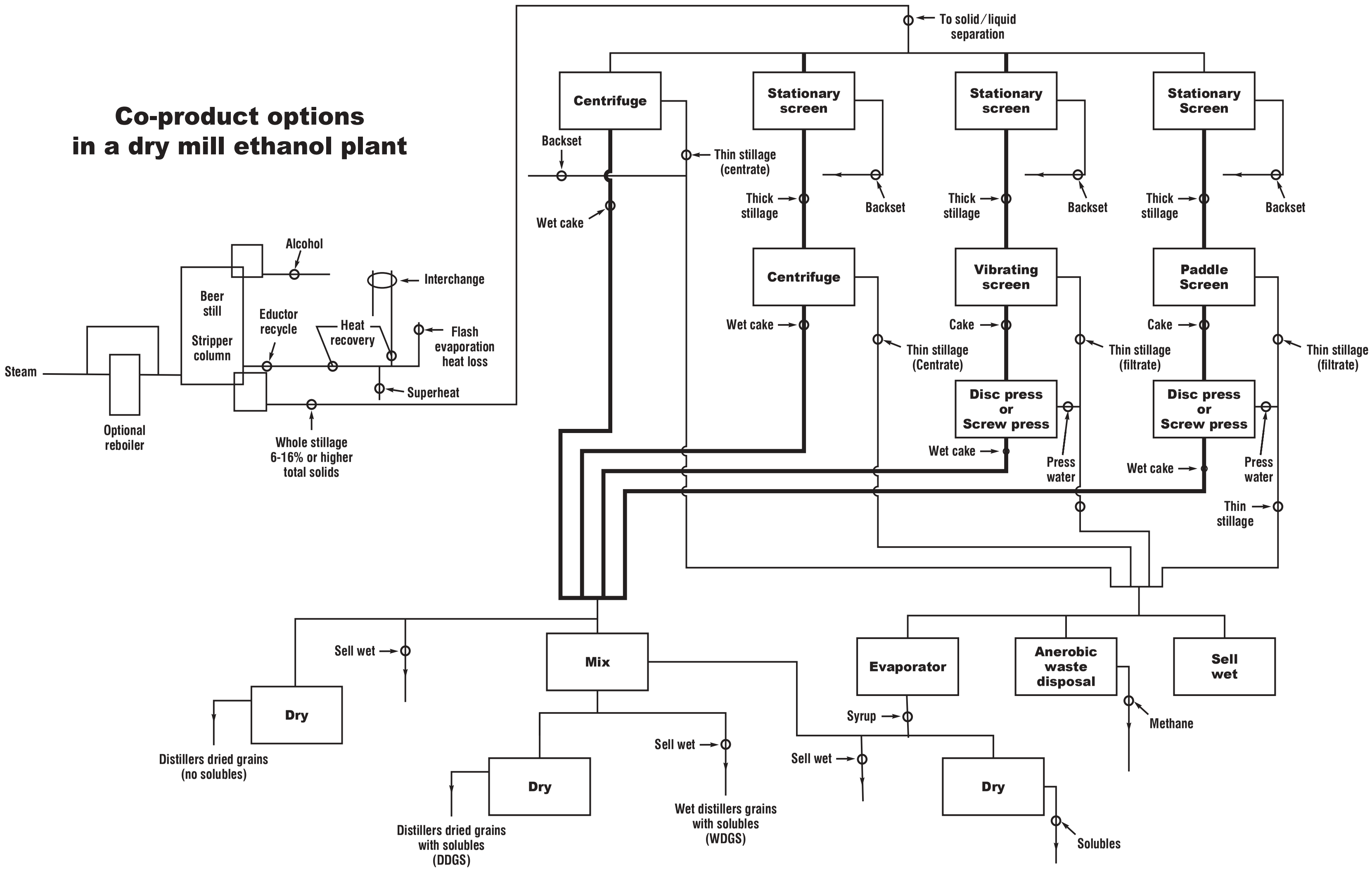

Co-product options
in a dry mill ethanol plant
To solid / liquid separation
Centrifuge
Stationary screen
Stationary screen
Stationary Screen
Backset
Thin stillage (centrate)
Wet cake
Thick stillage
Backset
Thick stillage
Backset
Thick stillage
Backset
Alcohol
Interchange
Beer still
Stripper column
Eductor recycle
Heat recovery
Flash evaporation heat loss
Steam
Superheat
Optional reboiler
Whole stillage 6-16% or higher total solids
Centrifuge
Vibrating screen
Paddle Screen
Wet cake
Cake
Cake
Thin stillage (Centrate)
Thin stillage (filtrate)
Thin stillage (filtrate)
Disc press or Screw press
Disc press or Screw press
Wet cake
Press water
Wet cake
Press water
Thin stillage
Mix
Evaporator
Anerobic waste disposal
Sell wet
Sell wet
Dry
Distillers dried grains (no solubles)
Dry
Distillers dried grains with solubles (DDGS)
Sell wet
Wet distillers grains with solubles (WDGS)
Syrup
Sell wet
Dry
Solubles
Methane

Figure 1. Co-products of ethanol production in a dry mill plant.

corn meal as the primary product and beverage spirit as a secondary product. Thick stillage developed into an excellent livestock feed ingredient. One gallon of thick stillage contains ~0.2 lbs. of protein. In the 1950s, farmers typically paid $8-10 per 1000 gallons for thick stillage.

Over time, the small corn milling operations were gradually replaced by larger plants producing beverage alcohol as the primary product. Storing and handling large quantities of thick stillage introduced problems as the distilleries increased in size. Whole stillage leaving distillation is a hot, acidic and viscous fluid with limited shelf life (Table 1). Rich in protein and other nutrients for bacterial growth, stillage will decompose rapidly depending on storage conditions.

Table 1. Whole stillage properties at the point of distillation.

Saturated liquid temperature	≥220°F
Total solids	6-16%
Dissolved solids	2-8%
pH	4.5 or less (corrosive)
General composition	Fiber, protein, fats/oil, water, yeast cells, acetic and lactic acid, other organic compounds including glycerol, minerals

For a variety of reasons drying processes developed in the 1990s to remove the water from the thick stillage. Drying eliminates the necessity to maintain a livestock operation. Shelf life is retained; and the need to handle an acidic and corrosive material precluded. The result is a valuable distillery co-product with less storage and transport volume needed.

Dryhouse design and co-product production

Reliability and maximum economic return are the two key issues that must be addressed in designing the dryhouse for modern ethanol and beverage alcohol plants. Process systems must operate with minimum downtime for cleaning and repair. Production must not be limited by problems in the dryhouse. To that end, each subsystem or unit operation listed must be reliable:

- whole stillage heat recovery
- whole stillage liquid/solid separation
- backset handling and heat preservation
- cake handling
- evaporation
- syrup handling
- drying
- dry solids handling and storage
- wastewater handling
- emission handling

Dryhouse reliability is also very much a function of fermentation efficiency in the front of the plant. Unconverted starch and sugar will quickly foul heat transfer surfaces in the dryhouse causing capacity reduction (poor heat transfer) and downtime.

Secondly, selection of the process system must address capital cost, revenues and operating costs such that the dryhouse remains a profit center. The capital cost for building the dryhouse depends on equipment selection as well as design capacity. Increasing the backset return to cooking and fermentation reduces the evaporative load on the dryhouse and thus the capital cost needed for construction.

Selection of equipment and the dryhouse process

In order to maximize the return on investment in dryhouse equipment, present value economic analysis should be completed for the various dryhouse options. Each co-product option carries with it operating costs, as noted in Table 2.

Equally important to operating costs are equipment reliability and longevity. All process materials in the dryhouse are corrosive and will attack mild carbon steel. The aggressiveness of the attack is somewhat dependent on moisture content. For example 30% total solids wet cake is more corrosive than 90% total solids DDGS.

Stainless steel 304 is the recommended material for most all applications. Copper bearing steel 'cor-ten' can be used in some of the less corrosive applications such as DDGS

Table 2. Co-product operating costs.

Co-product	*Amortization*	*Thermal energy*	*Electrical energy*	*Water and sewer*	*Labor*	*Chemicals and supplies*	*Marketing and overhead*
DDGS	Required	Required	Required	Required	Required	Required	Required
DDG:							
(dried wet cake)	Required	Required	Required	Negligible	Required	Required	Required
Solubles	Required	Required	Required	Required	Required	Required	Required
Thick stillage	Negligible	Not required	Negligible	Negligible	Negligible	Negligible	Negligible
Thin stillage	Required	Not required	Required	Negligible	Negligible	Negligible	Negligible
Wet cake	Required	Not required	Required	Negligible	Required	Negligible	Required
Syrup	Required	Required	Required	Required	Required	Required	Required

storage bins. Heated surfaces in some dryers can be carbon steel without corrosion problems. In some cases, particularly if sulfur is present, higher grades of stainless steel must be used.

Equipment selection by process system

WHOLE STILLAGE HEAT RECOVERY

Excepting vacuum distillation systems, whole stillage leaving distillation is a superheated liquid at approximately 220°F. If the first process following distillation is screening or centrifuging, then flash heat (220-212°F) is often lost. Available heat recovery processes include heat interchange with fluids to be heated (beer to stripper column, cook water) and eductor recycle to the base of the stripper column. It should be noted, however, that if the dryhouse includes evaporators and dryers, cooling of the whole stillage below the atmospheric boiling point causes the need for additional thermal energy later in the drying process.

LIQUID/SOLIDS SEPARATION

In order to produce any of the co-products (except thick stillage), the thick stillage (or whole stillage, see Figure 1) must be separated into a somewhat diluted liquid that will be partly used as backset and then can be further processed, and a bulk solid material typically called wet cake. Thick stillage (or whole stillage) total solids content in beverage plants typically runs 7-10% compared to 14% and higher for fuel ethanol plants. Beverage plants run ‘thinner’ beer primarily for quality reasons.

The 8% (or more) total solids in the thick stillage is composed of 3% (or more) dissolved solids plus the remaining suspended solids. Dissolved solids are defined as the solids that will pass through a No. 4 Whatman filter paper using a Buchner funnel and vacuum pump.

Good liquid/solid separation is key to running an energy efficient, low maintenance, dryhouse. As will be discussed later in this chapter, removing water in the dryer uses at least four to five times more energy than removing water in the evaporator. The amount of evaporation load that can be shifted to the evaporator is almost completely dependent on the concentration of suspended solids in the evaporator feed (dilute liquid noted above, also called thin stillage or centrate). Liquid/solid separating equipment determines this concentration.

Early dryhouse designs included combinations of screens and presses. One common design uses an inclined screen with moving blades or paddles. Stillage is pumped to the top of the screen. The thin stillage passes through the screen while the cake is guided down by the paddles and exits at the end of the screen, usually into a hopper to be fed into a press. These techniques result in reduced suspended solids content in thin stillage compared to the thick stillage. However with these systems, suspended solids in the thin stillage remains at levels (2.5% or more) that limit evaporator operation to syrup (evaporator product) levels of 25-28% total solids.

Centrifuges

Centrifuges gradually replaced many of the screen/press systems in the mid 1900s. Various

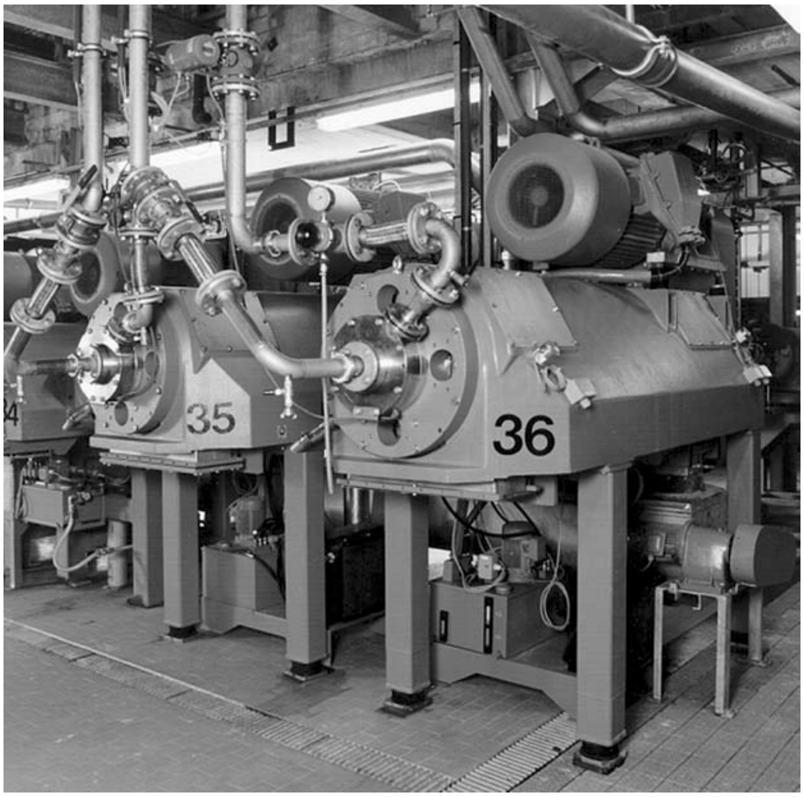


Figure 2. Centrifuge manufactured by Westfalia.

centrifuge configurations were installed; however, the horizontal solid bowl type with separating forces exceeding 3000 times the force of gravity performed best on distillery stillage.

Two companies, Sharples/Alpha Laval and Westfalia, have supplied centrifuge equipment to many ethanol and beverage spirit plants (Figure 2).

There are three continuous actions occurring in the centrifuge (reference Sharples procedures):

a. Slurry is introduced into the revolving bowl of the centrifuge through a stationary feed tube at the center of rotation. By centrifugal force, the solids (more dense than the liquid) are thrown to the wall of the bowl and the liquid forms a concentric inner layer in the bowl.

b. A helical screw conveyor located inside the revolving bowl rotates in the same direction as the bowl but at a slightly lower RPM. This conveyor moves the solids to one end of the bowl, where they are 'plowed up the beach' (narrowing of helical conveyor) and out of the liquid layer into discharge ports.

c. The clarified liquid is continuously removed by overflowing adjustable weirs (plate dams) inside the machine to the discharge point.

It is key to understand that the centrifuge must be fine-tuned at each plant location to reach optimum performance. Various on site adjustments are required and may take hours or days to reach design performance:

- The milling system at the front end of the plant affects particle size and centrifuge performance. Screen analysis from corn mills should be obtained and compared to other plants with good centrifuge performance.
- Depth of pond inside the centrifuge (plate dams are adjustable).
- Bowl RPM – example 2600.

- Delta RPM – example 4 to 6 RPM (back drive determines delta RPM between bowl and conveyor).
- Beach angle and length.
- Conveyor design including wear part protection (tiles and carbide wear parts).

Plant operators should monitor total, suspended and dissolved solids for the process streams entering and leaving the centrifuge. Low suspended solids in the centrate is the first objective. Values approaching 1% have been obtained depending on the plant. Low moisture in the cake is the second objective. Cake from a properly selected centrifuge typically runs 30% to 31% total solids.

Centrifuge installation

Manufacturers provide detailed and extensive installation instructions. However a few items are worth noting. Vents must be provided to atmosphere at each of the two discharge points. Considerable amounts of flash vapor discharges from the centrifuge. The liquid will cool somewhat (centrifuges are big ‘fans’) from entrance temperature. Secondly, the supporting structure for the centrifuge must handle static and dynamic loads and must be designed by a licensed structural engineer. These large, heavy, fast-rotating machines can impose dynamic loads in excess of three times the static load. Centrifuges are capital cost intensive and high maintenance machines. Spare parts including a spare bowl assembly are necessary for plants that run continuously. Lastly, it is interesting to note that screen/press systems will give a lower cake moisture (up to 38% total solids) than centrifuges. However, the high thin stillage suspended solids for the screen/press system shifts loads to the dryer and raises overall dryhouse operating costs in most cases. Depending on the application and selection of co-product types, the centrifuge will not always be the low cost life cycle option, however.

BULK SOLID CONVEYING AND STILLAGE PUMPING

Equipment must also be selected to move liquids and bulk solids in the dryhouse from process to process. Selecting bulk solids conveying equipment is the most challenging. Two methods are common: mechanical conveying and pneumatic conveying.

Mechanical conveyors including screw conveyors, belt conveyors, drag conveyors and bucket elevators are in service in dryhouses. Selection of the type of conveyor is dependent on several factors:

- Length of run
- Elevation change
- Properties of the bulk solids (moisture content, bulk density, angle of repose, fouling tendencies)
- Flow rate

Screw conveyors are commonly used for all applications with the exception of high flow rate systems. In these high flow systems, cost is a factor and often belt or drag conveyors are less expensive than large stainless steel screw conveyors. Bucket elevators have limited application in dryhouses due to the properties of most of the bulk solids, which lead to fouling of the buckets. However, bucket elevators have been used successfully in handling 90% total solids material.

Pneumatic conveying becomes a viable method when conveying distances are excessive and/or elevation change is large, however, certain design issues must be addressed:

- Limit the applications to low moisture bulk solids. DDGS, solubles and DDG (no solubles) are all successfully transferred pneumatically.
- Dryhouse bulk solids generally contain fiber and are abrasive. Use flat back ells and conservative air:solids ratios.
- Pneumatic conveying introduces the need for air/solids separation at the end point. This requires the use of cyclones, bag houses and vent filters and introduces an air emission point.

These considerations may eliminate viable pneumatic conveying at some locations.

Stillage pumping systems in the dryhouse do not require exotic, extremely costly equipment. Thick stillage and thin stillage can be pumped with centrifugal pumps using an 'open impeller'. Syrup should be pumped with a positive displacement pump (lobe type is suitable).

All piping systems in the dryhouse should be free of internal dead spots. Reduced port ball valves should not be used. Blind Ts should be avoided. Butterfly valves, ball valves, full port mag-meters and 'DU-O' check valves are all suitable. Rupture discs are preferred to relief valves due to fouling.

EVAPORATION

Thin stillage containing 1% or more suspended solids and 3% or more total solids can be concentrated in an evaporator to yield a product (syrup) that ranges from 25 to 50% total solids. Evaporators allow reuse of thermal energy by taking advantage of the enthalpy of evaporated vapor at reduced pressure, or by vapor compression (thermal or mechanical) and then using this evaporated vapor for further evaporation.

Evaporators find applications in many industries. Only certain types of evaporator configurations however, have been used successfully by ethanol and beverage spirit plants. Choices must be made among:

- Vessel configuration (see Figure 3).
- Forced circulation *vs.* natural circulation.
- Rising film tubular *vs.* falling film tubular.
- Plate equivalents of tubular evaporators.
- Multiple effect vacuum with thermal recompression *vs.* mechanical vapor recompression turbine drive or mechanical vapor recompression motor drive (Figures 4 and 5).
- Combination evaporator/distillation systems.

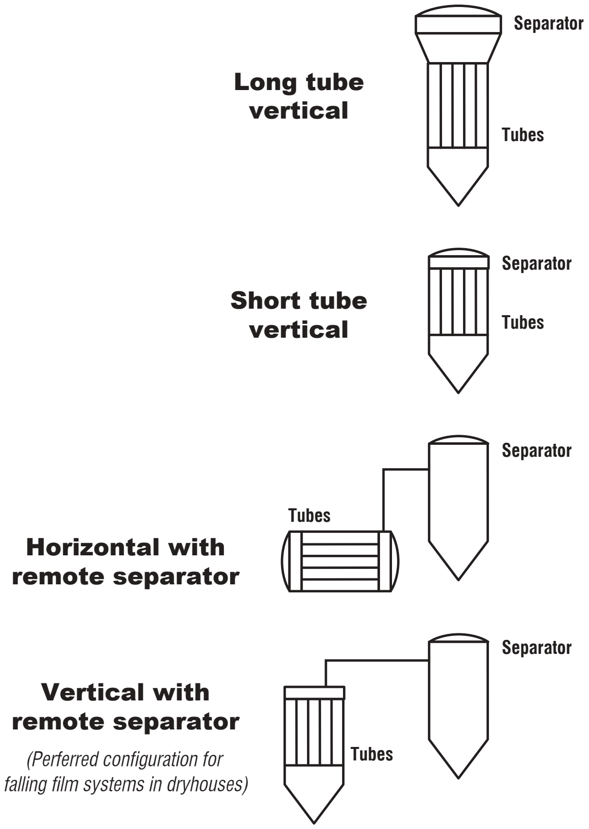

Separator
Long tube vertical
Tubes
Separator
Short tube vertical
Tubes
Separator
Tubes
Horizontal with remote separator
Separator
Vertical with remote separator
(Perferred configuration for falling film systems in dryhouses)
Tubes

Figure 3. Evaporator vessel configuration options.

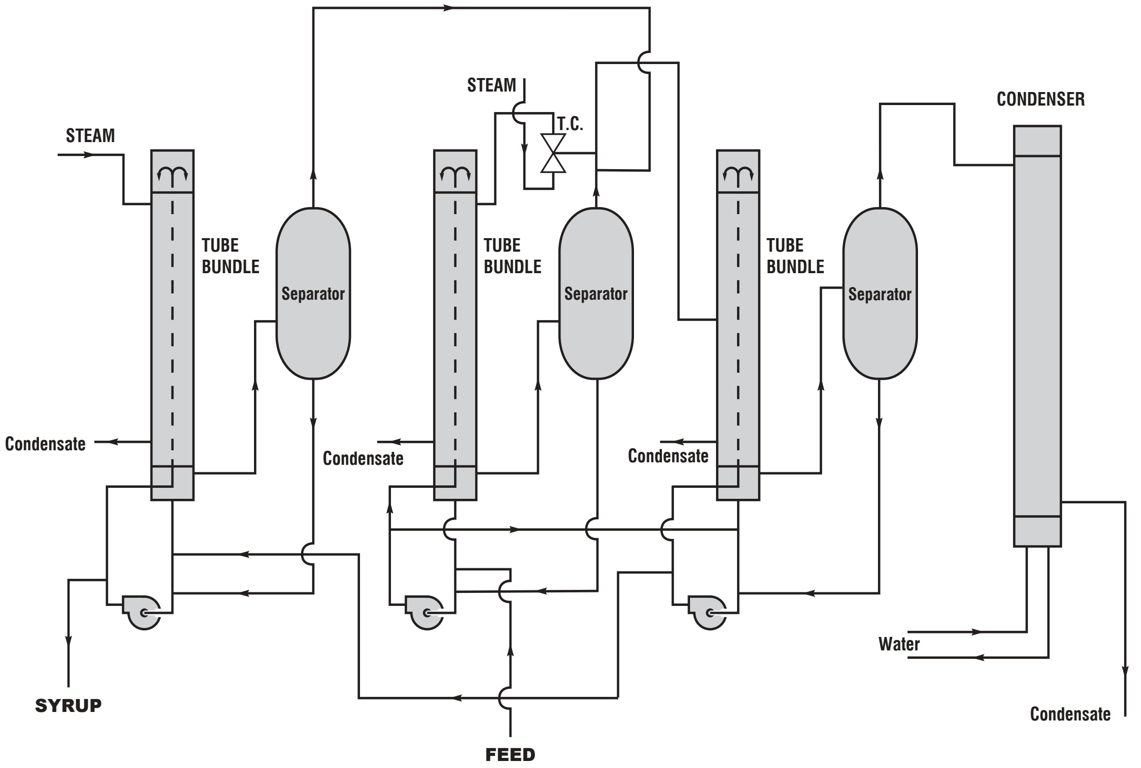

STEAM
TUBE
BUNDLE
Separator
Condensate
SYRUP
STEAM
T.C.
TUBE
BUNDLE
Separator
Condensate
FEED
TUBE
BUNDLE
Separator
Condensate
CONDENSER
Water
Condensate

Figure 4. Falling film multiple effect evaporation with thermal recompression.

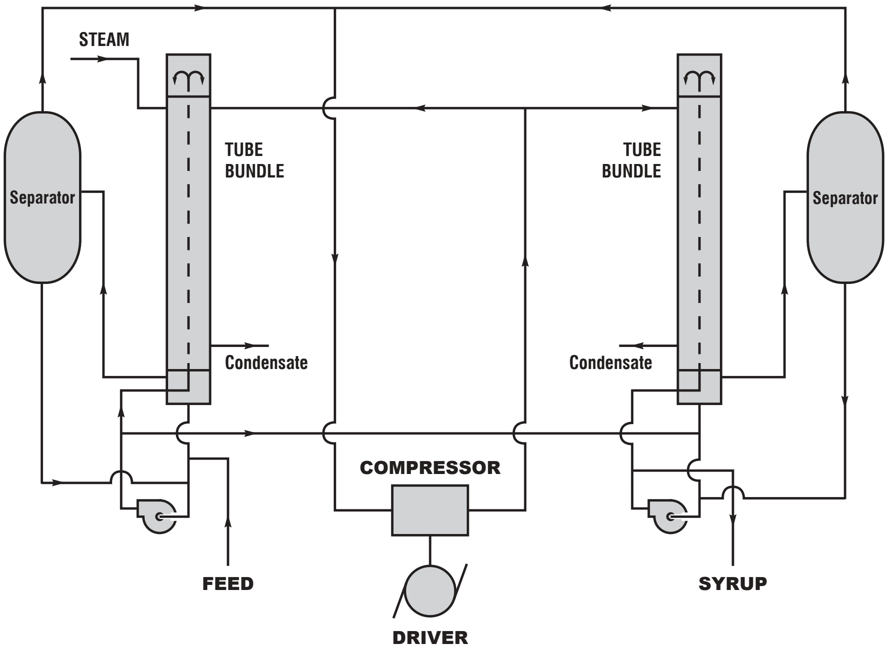

STEAM
Separator
TUBE
BUNDLE
Condensate
FEED
COMPRESSOR
DRIVER
TUBE
BUNDLE
Condensate
Separator
SYRUP

Figure 5. Falling film mechanical vapor recompression evaporator.

Rising film and plate equivalents of tubular evaporators have failed and/or caused excessive maintenance in ethanol and beverage spirit plants and should be avoided. Forced circulation falling film tubular vessel designs are the most user-friendly designs for plant dryhouses. The key to falling film systems is the design of the distribution head at the top entry to the heat exchanger. Each tube should receive the same design GPM. Multiple effect and recompression systems are equally acceptable for the dryhouse. The final choice depends on life cycle economics.

Additional evaporator design considerations

Ethanol plant evaporators are often integrated with the distillation and molecular sieve systems to save energy.

Thin stillage reboiler. The first effect of the evaporator becomes the reboiler for the stripping column. This saves energy compared to the use of live steam on the stripper and is more user-friendly (less fouling) than using a whole stillage reboiler on the stripper.

Hot vapor condenser as first effect of evaporator. The first effect of the evaporator replaces one of the condensers off the rectifying column or off the molecular sieve – also an energy saver.

Optimum run cycle should be balanced vs. syrup concentration

Evaporator cleaning is expensive, causing lost production and the need for energy input without product output. Caustic and other chemical costs are also incurred. Each plant should develop the optimum evaporator run time before cleaning by balancing syrup concentration, operating costs and cycle times between cleanups. Overall costs to produce 35% syrup may exceed costs to produce 30% syrup because of shortened cycle times at 35% syrup.

Energy costs vary depending on evaporator design; and as with other plant systems, must be analyzed based on capital cost/operating cost trade offs. Typically, a triple effect thermal recompression evaporator will evaporate 4.1 lbs vapor per pound of steam consumed. However, four effect units may approach 6 to 7 lbs vapor per pound of steam. The cost of the fourth effect must be justified by the increased thermal efficiency. Likewise, depending on heat transfer surface provided, a large mechanical vapor recompression unit might evaporate 100,000 lb/hr of vapor with a power requirement of 1000 kW. Less efficient units would have a lower first cost.

DRYERS

Dryhouse reliability and operating cost/return issues hinge as much on the selection of the dryer as any other system in the facility. Dryers are major users of energy and the largest source of air emissions in many ethanol and beverage spirit plants. Further, dryer failure will shut the plant down and in some cases dryer failures are not quickly remedied. Selection of the dryer depends on various process and operating considerations:

- Energy available (gas, oil, steam).
- Cost of energy options ($/BTU).
- Co-product to be dried (DDGS, syrup).
- Capital cost/operating cost tradeoffs (dryer efficiency *vs.* dryer cost).
- Corrosion.
- Operating time between shutdowns for cleaning.
- Preheat methods for air including heat recovery (consider flue gas, steam condensate, recycle exhaust).
- Environmental issues (dryer emissions)
  - scrubber
  - thermal oxidizer
  - regenerative thermal oxidizer
  - remove particulates, VOCs, odor

Types of dryers

As with evaporators, ethanol and beverage spirit plants have used different dryer types dating to the early 1900s (Table 3).

Table 3. Dryer types, energy sources and co-products produced.

Type/description	*Energy source*	*Typical co-product*
Steam tube (rotating drum)	Steam	DDGS
Steam tube (rotating tube bundle)	Steam	DDGS
Direct fire (rotating drum)	Gas, oil	DDGS
Flash dryer	Steam, gas, oil	DDGS
Drum/dehydrator	Steam	Solubles
Spray drier	Steam, gas, oil	Solubles

Steam tube rotating drum

This dryer is one of the original designs for distillery dryhouses and many installations around the world exist today. Steam tubes (typically 3 or 4 inch pipe) are fixed and located in one or more concentric rings inside the drum. Drum diameters may exceed 10 feet, and drum lengths are often 60 feet or longer. Steam enters a distribution head and condensate leaves from the same end. As with all dryers, a well mixed, properly conditioned feed is necessary to prevent fouling (see following discussion on feed conditioning). Each vendor uses in-house design techniques to lift and move the grain through the rotating drum. Efficiencies without air recycle average 0.6 to 0.7 lbs evaporated per pound of steam. Inlet air temperatures may approach 300°F with exit temperatures of approximately 200°F.

Steam tube rotating tube bundle

This is a variation of the steam tube rotating drum. In the rotating tube bundle, the drum is fixed and the tube bundle rotates. More heat transfer surface is located in a smaller drum compared to the rotating drum. Efficiencies are similar to the rotating drum. Feed conditioning to prevent fouling is critical to the rotating tube bundle type because of the close clearances between the tubes.

Direct fire rotating drum

Direct fired rotating drum dryers also have a long history of service in the ethanol and beverage spirit industries. These dryers have a fixed furnace section for heating the drying and conveying air stream, followed by the feed introduction point, which is in turn followed by the rotating drum. Drum designs vary, with both single pass and multiple pass units available.

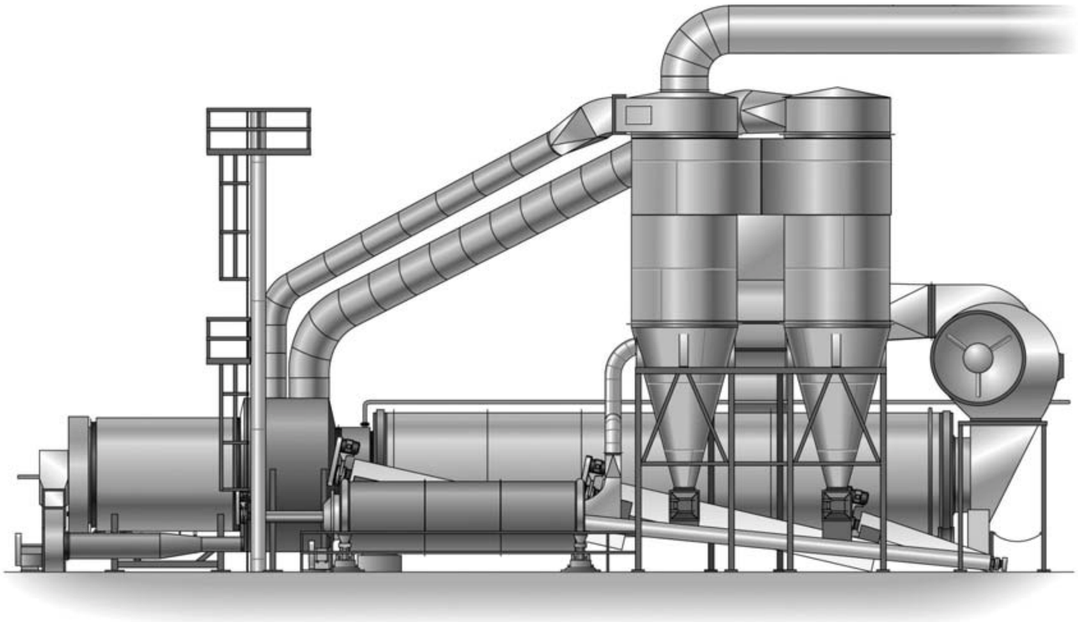


Figure 6. Typical Ronning dryer system.

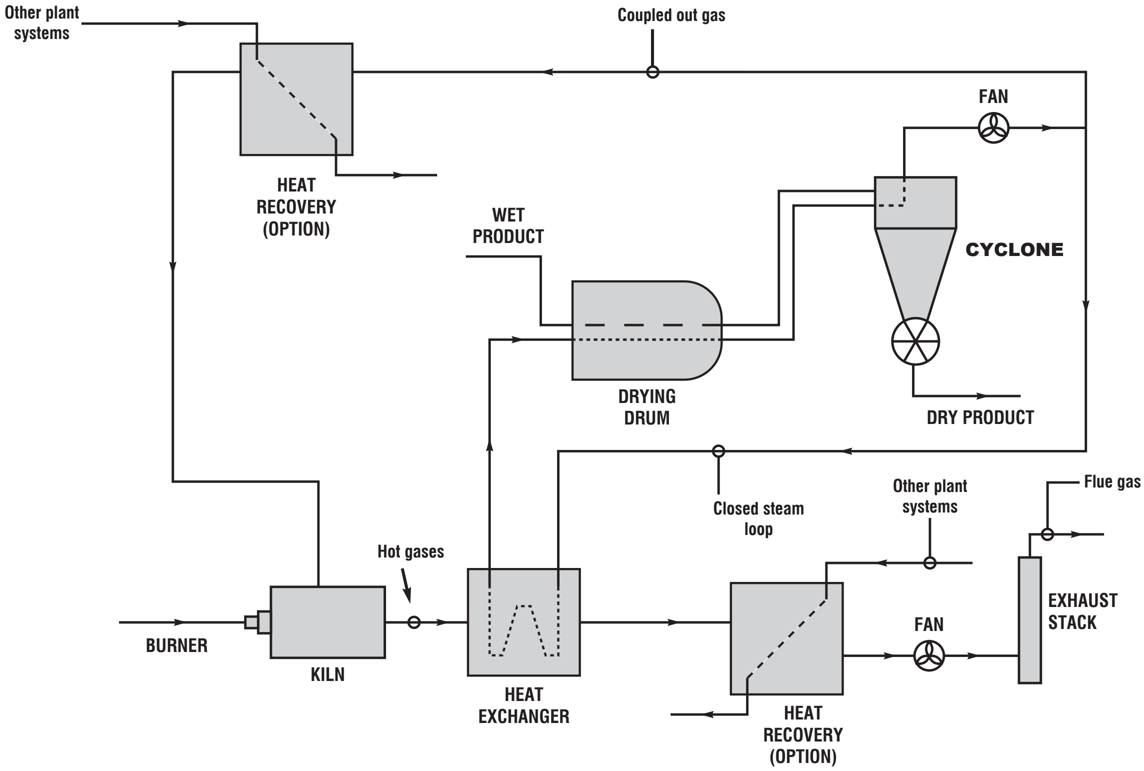

Other plant systems
Coupled out gas
FAN
HEAT RECOVERY (OPTION)
WET PRODUCT
CYCLONE
DRYING DRUM
DRY PRODUCT
Closed steam loop
Other plant systems
Flue gas
Hot gases
EXHAUST STACK
FAN
BURNER
KILN
HEAT EXCHANGER
HEAT RECOVERY (OPTION)

Figure 7. Schematic of the ecoDry system for DDGS.

Unlike steam tube dryers, direct fire rotating drum dryers must include cyclones to separate the dry product from the conveying air stream. Modern versions of these dryers include air recycle to capture and reuse energy.

Depending on design, inlet air temperatures may vary from 600 to 1200°F. Exit temperatures are typically 200°F. The elevated inlet temperatures, compared to steam tube systems, will result in additional dryer stack emissions.

Efficiencies are similar to steam tube systems, again depending on design. One direct fire rotating drum drying system by Ronning Engineering Co., Inc. shows energy use of 1350 BTU per pound of water evaporated. A typical Ronning Dryer System is illustrated in Figure 6. A schematic of a direct fire rotating drum system with air recycle and incineration of emissions by ecoDry is illustrated in Figure 7.

Flash dryers

Flash drying technology utilizes a stack or duct system instead of a rotating drum for contact between the hot drying and conveying air stream and the grain. Other features are similar to the direct fire rotating drum including: fixed furnace, feed entry point, cyclones and possibility of air recycle. Some vendors use the term ‘ring dryer’ instead of flash dryer.

Drying syrup into solubles

Drying evaporator syrup is more challenging than drying the mixed wet cake/syrup combination. The syrup is a viscous liquid prone to foul contact surfaces. In contrast, the mixed wet cake/syrup combination is a solid and can be conditioned with DDGS recycle before drying. In addition, dry solubles are extremely hygroscopic and difficult to handle.

Two technologies, the drum dehydrator and the spray dryer, have been used to produce dry solubles. In drum dehydrators, the syrup is deposited as a film on the outside of a heated (steam) rotating steel drum. The resulting dry sheet is continuously removed by a blade and then milled or ground into a powder. Drying syrup with a spray dryer is not common practice. Difficulties include reaching the very low moisture required for solubles (often 4-5%) without fouling the dryer. The resulting product can be hydroscopic and difficult to handle. However, Alltech has successfully used this technology and continues to improve the process at two plants.

Feed conditioning for dryers

As noted above, wet cake moisture is typically 70% and syrup moisture may be as high as 75%. Feeding this mixture without conditioning to most dryers will cause rapid fouling and in some cases may lead to dryer fires. The dryer feed should be conditioned and the moisture content decreased by recycling some dry product and mixing it with the wet cake and syrup. Feed moisture contents of 25 to 30% will enhance performance in most DDGS dryers. The exceptions are drum dehydrators and spray dryers, which are fed syrup 'as is'.

Selecting the wet cake/syrup/recycle mixer is again a trade-off between capital cost and operating performance. Systems that have been used include:

- U-Trough screw conveyor with mixing blades.
- Pugmill/double screw conveyor with mixing blades (horizontal).
- Vertical enclosed screw conveyor.
- Solid/liquid continuous in-line blenders.
- Ribbon blenders.

Considering mixing efficiency, horsepower and corrosion/erosion control, the double screw pugmill is a good selection.

WASTEWATER AND EMISSIONS

Wastewater

Evaporator condensate (water removed from evaporator feed plus in some cases mixed in steam condensate) and CIP (clean-in-place) systems are the two primary sources of wastewater from ethanol and beverage spirit dryhouses. Many ethanol plants recycle all or part of the evaporator condensate back to the cooking system. Beverage plants do not recycle due to quality concerns. The recycled condensate must be conditioned with chemicals and/or make up water to prevent contamination and unacceptable pH levels.

Whole stillage, thick stillage and thin stillage (evaporator feed) all have a BOD (Biological Oxygen Demand) of over 20,000, compared with approximately 250 for domestic waste. Evaporator condensate BOD will range from 1000 to as high as 5000. The condensate is acidic (usually lactic and acetic acids) with a pH of 4.0 or less. EPA direct stream discharge laws usually limit BOD to 15 to 30.

Various wastewater treatment systems are in place in most ethanol plants and virtually all beverage plants to condition some or all of the wastewater to acceptable levels. Requirements vary from location to location and also depend on access to municipal sewers. When municipal sewers are an option, dryhouse wastewater is pre-treated before discharge to the sewer. Wastewater pre-treatment requirements also vary, but a reduction in BOD to 200 would be typical.

Wastewater systems must address the following:

- BOD reduction.
- Suspended solids reduction.
- Temperature.
- Acidity.
- Reduction of other regulated contaminants.
- Sludge handling and disposal.

Wastewater treatment options include:

- Aerobic digestion with lagoons or tanks.
- Sequential batch reactors.
- Anaerobic conversion to methane.
- Land application.

Dryhouse air emissions

Sources for air emission in dryhouses include the dryer stack, cyclones, bag houses and tank vents. Until about 1999, the US Environmental

Protection Agency (EPA) was primarily interested in particulates and the abatement equipment of choice was normally a wet scrubber. However, tests in Minnesota at an ethanol plant revealed volatile organic compound (VOC) and carbon monoxide (CO) emissions from a direct gas fired DDGS dryer that exceeded permissible levels.

In many states, the EPA now requires incineration of VOC, CO and particulates using thermal oxidizers. The design must provide for a specified temperature (usually 1400°F) and a set contact time. Two gas fired oxidizer systems are being used by the industry:

Regenerative thermal oxidizers This system utilizes two beds. One bed is online while the second preheats the incoming air using heat trapped in the preceding cycle when it was online. Vendors state that a 95% recovery of energy is possible. However, fouling occurs (dryer stack emissions may include oils and grease from the corn) and 'bakeouts' are necessary.

Thermal oxidizer with waste heat boiler. This system eliminates the heat retaining bed and uses the hot air discharge from the oxidizer to generate steam for the plant with a waste heat boiler. Vendors state that overall thermal efficiency for this system is comparable to pre-oxidizer days when steam was generated by gas fired boilers and dryer stack emissions were not thermally abated.

Storage options for co-products

Wet co-products including thick stillage, wet cake and syrup have a limited shelf life. Inventory at the dryhouse for these co-products should not exceed a few days. DDGS and solubles, however, can be stored for weeks if conditions are suitable.

Bridging and plugging are problems for DDGS and solubles. Storage in high humidity conditions aggravates the bridging. Storage in bins and hoppers should be limited to bins with 'live bottoms' – typically multi-screw feeder types. Most ethanol plants store DDGS on concrete pads within a weather-tight building. Sanitation procedures must be in place to prevent spoilage and infestation.

Control systems

With the exception of clean up procedures, dryhouse operations are continuous processes. A properly designed, distributed control network will provide years of reliable operation.

One operator monitoring the network can responsibly control centrifuges, evaporators, dryers, conveyors and accessories. One additional operator is usually required to handle batch operations including CIP, chemical additions to wastewater, etc.

Chapter 27

Ethanol production and the modern livestock feed industry: a relationship continuing to grow

KATE A. JACQUES

North American Biosciences Center, Alltech Inc., Nicholasville, Kentucky, USA

The rapid increase in production of ethanol in North America is paralleled by the rise in co-products produced, particularly those from the dry milling ethanol process. While there is undoubtedly great potential for new products based on various fractions of stillage, true realization of that potential will take both time and considerable industrial creativity. At present, the immediate issue is finding ways to maximize return for the co-products currently produced, which is mainly distillers dried grains with solubles (DDGS).

The primary market for DDGS today is livestock feeding. Of the >3.5 million mT of DDGS produced in North America (figures for the year 2000), around 800,000 mT are exported and the remaining 2.7 million mT are used in US and Canadian animal feeds (Markham, 2003; Figure 1). If ethanol production from dry mills, and consequently DDGS, production is to double as predicted in a few short years, then clearly we must actively pursue ways to increase its utilization if DDGS is to maintain its valuable role in the economics of ethanol production.

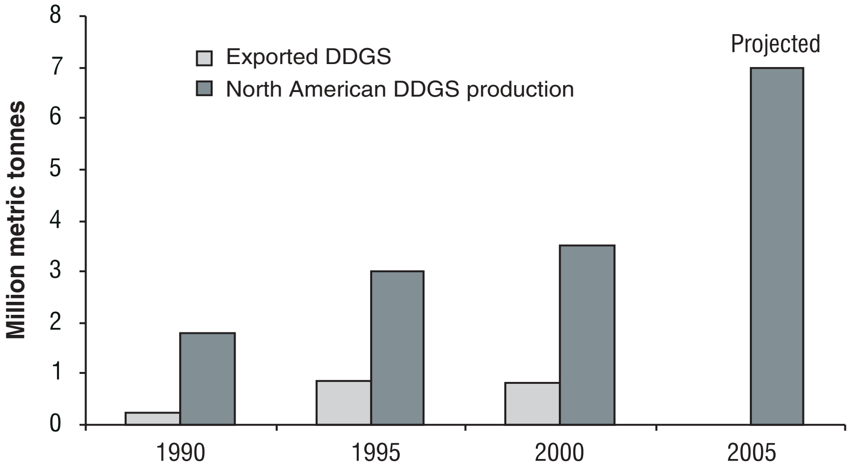


Figure 1. Production and exports of DDGS in North America (adapted from Markham, 2003).

There is a growing amount of animal nutrition research in recent years that illustrates that DDGS could play a much larger and more useful role in modern food and companion animal nutrition. Work with dairy and beef cattle, pigs, poultry and even fish shows that the 'new' DDGS has higher nutrient content, greater flexibility in formulations, can be used at higher inclusion levels and in general provides a cost-effective means of meeting the goals of modern nutrition. While the impact of this work is growing, two key issues must be addressed if DDGS use in the feed industry is to increase significantly. One is a greater understanding on the part of the ethanol industry of what today's feed industry values in ingredients. The second is a change in the feed industry perception of DDGS.

The contemporary animal feed industry is very different from the industry in the 1970s and 1980s when much of the initial nutrition research with DDGS was conducted. At that time, the primary approach to DDGS use was a straightforward replacement of other dietary protein sources. Today both the animal and the context in which it is fed have changed. Modern livestock genetics have given us higher performance expectations for meat, milk and egg production; nutritional knowledge has grown, and consequently diet formulations have gotten more complicated. Feeding goals have changed as well. There is a focus on animal health aspects of nutrition as well as performance, a general move away from animal protein sources in feeding programs in many parts of the world and the environmental impact of food animal production is a critical consideration. Distillery co-products can have an impact in all of these areas; and getting that information into the marketplace can increase overall co-product usage to mutual benefit.

'New generation' DDGS *vs* old feed industry perceptions

Much of the feed industry still perceives DDGS as an 'alternative protein source' commodity with limitations in quality, consistency or composition that restrict its use as a feed ingredient. Much of this impression, however, stems from the DDGS produced using technologies common 20 to 30 years ago, but is perpetuated by industry references that provide 'book values' based on 'old DDGS'. For example, energy content in DDGS from modern ethanol plants is higher than in 'old' DDGS; and both are higher than values published in the most recent edition of Nutrient Requirements of Swine (Table 1, Shurson *et al.,* 2003). Similarly, values for total protein and certain limiting amino acids, particularly lysine, are notably higher in new DDGS (Table 2).

Dated reference values underscore the need for suppliers of DDGS to provide complete analytical data. Diet formulation software incorporates all nutrient fractions of each ingredient; and reliance on 'book values' of any sort by nutritionists is avoided. Part of the quality control program for feed manufacturers is keeping ingredient composition updated such that fluctuation in nutrient content of an ingredient will not result in a change in animal performance.

The lighter or 'golden' color of DDGS from modern plants is desirable in that it indicates less heat damage to nutrients, which particularly affects amino acid digestibility. The dark color of earlier DDGS often indicated overheating and carmelization of nitrogenous components and sugars that escaped fermentation. In modern processes less sugar escapes fermentation, stillage viscosity tends to be lower, and more

Table 1. Comparison of energy values for DDGS (dry matter basis)[1].

	New DDGS (Calculated)	*New DDGS (Trial average)*	*Old DDGS (Calculated)*	*DDGS (NRC, 1998)*
Digestible energy, kcal/kg	3965	4011	3874	3449
Metabolizable energy, kcal/kg	3592	3827	3521	3038

Corn: DE (kcal/kg) = 3961, ME (kcal/kg) = 3843

[1] Shurson *et al.*, 2003

Table 2. Comparison of amino acid composition of DDGS (dry basis) in old generation DDGS, new generation DDGS, and values published in NRC (1998)[1,2].

	Old generation DDGS	*New generation DDGS*	*DDGS NRC (1998)*
Arginine, %	0.92 (18.7)	1.20 (9.1)	1.22
Histidine, %	0.61 (15.2)	0.76 (7.8)	0.74
Isoleucine, %	1.00 (9.1)	1.12 (8.7)	1.11
Leucine, %	2.97 (12.4)	3.55 (6.4)	2.76
Lysine, %	0.53 (26.5)	0.85 (17.3)	0.67
Methionine, %	0.50 (4.5)	0.55 (13.6)	0.54
Phenylalanine, %	1.27 (8.1)	1.47 (6.6)	1.44
Threonine, %	0.98 (7.3)	1.13 (6.4)	1.01
Tryptophan, %	0.19 (19.8)	0.25 (6.7)	0.27
Valine, %	1.39 (2.3)	1.50 (7.2)	1.40

[1] Values in parentheses are coefficients of variation among ethanol plants.

[2] Shurson *et al.*, 2003

water can be removed by evaporation, which reduces heat input in the dryhouse. Ergul *et al.* (2003) found that color was a good predictor of amino acid digestibility in chickens and that a lighter, more yellow color was associated with higher lysine, cystine, and threonine digestibility.

Nutritional value of DDGS

PROTEIN, ENERGY AND PHOSPHORUS CONTENT

Fermentation of the starch in cereal grains, which constitutes about 66% of the weight, concentrates remaining nutrients (fat, protein and minerals) by approximately a factor of 3. In nutritional terms, this makes DDGS a good source of protein, energy and phosphorus, the three most expensive nutrients added to livestock diets. For corn, which is normally 9-10% crude protein, resulting DDGS become 28-30% (or more) crude protein. Though the energy in starch is removed during fermentation, the fat remains. Concentration, added to the fact that fat contains three times the energy as starch, brings DDGS energy content nearly equal to that of corn. This indicates that DDGS can be used to replace a portion of the corn or other energy grain. The fibrous fraction of corn is concentrated to 7-8% crude fiber in corn DDGS. Phosphorus, notably low in corn (0.28%) is raised to 0.7-0.8% in DDGS. More importantly, the phosphorus in DDGS becomes 'available' for digestion following ethanol fermentation. Pigs, poulty and other monogastric species lack the phytase enzyme needed to hydrolyze phosphorus from the phytin form in which it exists in cereal grains (Figure 2). General composition of grain and DDGS for corn, wheat and grain sorghum are in Table 3.

Table 3. Composition of grains and DDGS made from them.

	Corn		*Sorghum*		*Wheat*	
	Grain[1]	*DDGS[2]*	*Grain[1]*	*DDGS[2]*	*Grain[1]*	*DDGS[2]*
Dry matter, %	89	88.9	89	90.31	88	92.48
Crude protein, %	10.3	30.6	9.2	30.3	13.5	38.48
Crude fiber, %		8.8		10.7		6.01
Crude Fat, %	6.7	10.9	2.9	12.5	2.0	8.27
Calcium, %	0.05	0.06	0.03	0.10	0.06	0.15
Phosphorus, %	0.43	0.89	0.29	0.84	0.37	1.04

[1] NRC, 1998

[2] cited at http://*www.ddgs.umn.edu/*

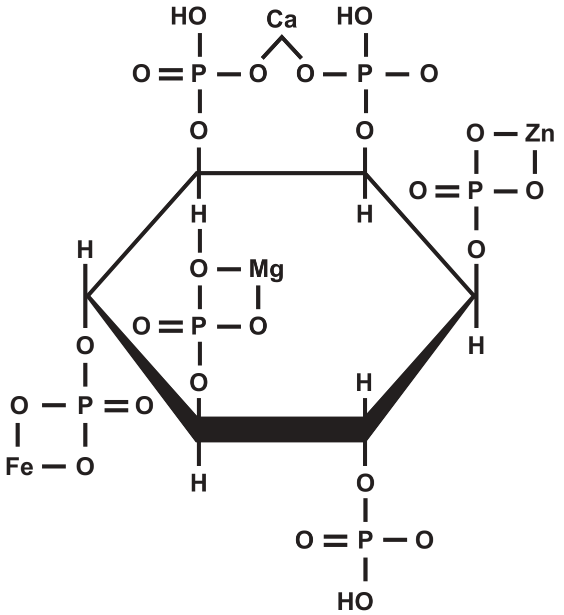


Figure 2. Structure of phytin phosphorus in cereal grains.

CONTRIBUTION OF SOLUBLES VS GRAINS

The amounts and forms of nutrients vary between grains and solubles; and the differences in quantities of solubles added back to grains is one potential source of variation in DDGS nutrient content between ethanol plants. Spent grains are higher in protein than solubles and contain almost all the fiber. Solubles, in contrast, contain most of the fat, phosphorus and other minerals. As such, variations in the amounts of solubles added can have the biggest potential impact on the overall energy and phosphorus content of DDGS (Knott *et al.* (2001).

Knott *et al.* (2001) examined grains and solubles from six Minnesota ethanol plants to determine nutrient variability in these fractions over a 3-week period (Table 4). Nutrient content of grains generally varied less than did solubles, and crude fat content of both grains and solubles varied more than any other nutrient.

VARIATION IN DDGS IMPACT ON NUTRITIONAL VALUE

Knott *et al.* (2001) also noted that some plants had much more variation in particular nutrients between batches than others; and the authors calculated the impact this would have on protein, fat or phosphorus of DDGS based on inclusion of 100% of the solubles (Table 5). In the plant with least variation, crude protein content varied only 2 percentage points, however it varied by 10 percentage points in the plant with most variation. Coefficients of variation exceeded 20% for fat and phosphorus content in this plant as well. From a nutritionist's standpoint, the two batches of DDGS represented by the minimum and maximum from that source vary markedly in value in terms of cost per nutrient unit or potential use in the least cost formulation. While percentage differences may appear small, they can have a large impact on the amounts of other, more expensive ingredients DDGS might replace. This, in turn, affects the value of DDGS to the feed manufacturer.

CAN WE INCREASE USE OF DDGS IN DAIRY AND BEEF CATTLE?

Currently beef and dairy cattle consume about 80% of the DDGS sold in North America (Figure 3, Markham, 2003). DDGS is of value both in terms of energy and protein for meat and milk production, contributes digestible fiber and contains nutritionally meaningful amounts of phosphorus.

Protein nature and quality

Traditionally, most DDGS has been used in cattle for its 'by-pass' protein value, which allows reduction of more expensive soybean meal (eg) in the diet. Ruminants are unique in that the first compartment of the digestive tract is an anaerobic fermentation, which allows the digestion of fiber by symbiotic bacteria in the rumen. As such, part of the animal's protein/amino acid supply comes from microbial protein and part from feed protein that escapes or 'by-passes' rumen microbes and is digested enzymatically in the small intestine. High performance dairy and beef cattle diets are balanced to supply both rumen-degradable and rumen escape, or rumen-undegraded protein. The general goal is to let rumen microbes upgrade the biological value of poor quality nitrogen sources (like yeast, rumen bacteria can use simple N sources like urea or other FAN) by

Table 4. Average, minimum, maximum, and mean nutrient values (dry matter basis), of grains and solubles fractions from six Minnesota ethanol plants[1].

	Average	*Minimum*	*Maximum*
Grains fraction			
Dry matter, %	34.3	33.7	34.9
Crude protein, %	33.8	31.3	36.0
Crude fat, %	7.7	2.1	10.1
Crude fiber, %	9.1	8.2	9.9
Ash, %	3.0	2.6	3.3
Calcium, %	0.04	0.03	0.05
Phosphorus, %	0.56	0.44	0.69
Solubles fraction			
Dry matter, %	27.7	23.7	30.5
Crude protein, %	19.5	17.9	20.8
Crude fat, %	17.4	14.4	20.1
Crude fiber, %	1.4	1.1	1.8
Ash, %	8.4	7.8	9.1
Calcium, %	0.09	0.06	0.12
Phosphorus, %	1.3	1.2	1.4

[1] From Knott *et al*., 2001

Table 5. Predicted nutrient content and coefficients of variation in DDGS from plants with the least or most variation in protein, fat or phosphorus content[1,2].

	Plants with least variation	*Plants with most variation*
Crude protein		
Minimum, %	29.0	19.8
Maximum, %	31.2	29.3
Mean, %	29.9	25.8
CV	3.57	20.8
Crude fat		
Minimum, %	11.0	7.9
Maximum, %	12.6	11.5
Mean, %	12.0	9.8
CV	7.00	43.58
Phosphorus		
Minimum, %	0.73	0.63
Maximum, %	0.82	0.96
Mean, %	0.70	0.76
CV	7.64	22.44

[1] From Knott *et al*., 2001

[2] DDGS nutrient content predicted assuming 32 gallons of syrup added/minute, 79.63 lbs of dry syrup added/minute, 40.63% solubles and 59.37% grains in DDGS on a dry matter basis.

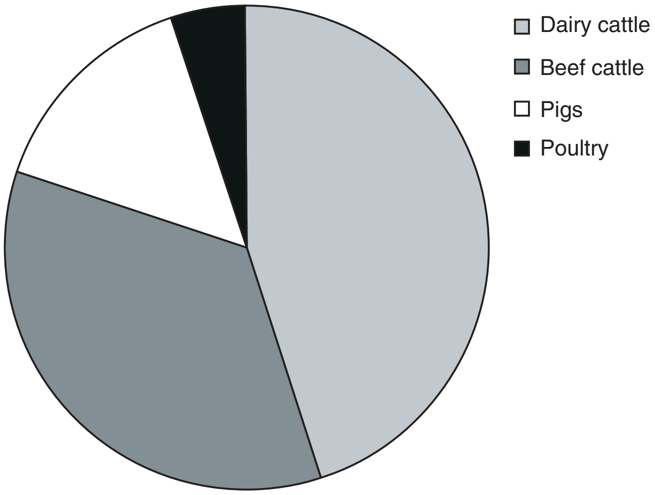


Figure 3. Utilization of DDGS by the North American livestock feed market (Markham, 2003).

converting it to microbial protein. On the other hand, some feed protein sources are higher in biological value (amino acid profiles useful to the animal) than microbial protein and of greater benefit to the animal if they 'by-pass' rumen fermentation. Because it has already been through fermentation, over 50% of DDGS protein is 'by-pass' protein (Schingoethe, 2001), which compares favorably with other available sources (Table 6).

Table 6. Comparison of the crude protein and rumen-undegradable protein contents of various feedstuffs.

	Crude protein (%)	*Rumen undegradable protein (%)*
Forage sources		
Alfalfa hay	19	28
Alfalfa silage	19	23
Corn silage	9	31
Distillery co-products		
Corn DDGS	28	54
Oilseed meals		
Soybean meal	47	35
Canola meal	42	28
Sunflower meal	26	26
Animal sources		
Fishmeal	67	60
Feather meal	89	71
Blood meal	92	82
Meat and bone meal	42	61

While substitution of DDGS for other protein ingredients is most common, use of DDGS to replace energy sources in ruminants may provide benefits to rumen/animal health as well as boost dietary inclusion rates. Starch overload in the rumen of a dairy cow or beef finishing animal is a common cause of acidosis, low milk fat content and variation in feed intake and performance. Distillers grains (wet or dry) are similar in energy content to the original grain, but the energy value is contributed primarily by concentration of the fat along with some digestible fiber. In addition, bypass protein has a higher energy value than the same protein degraded in the rumen. Lower incidence of acidosis and the impact of energy level and form have been suggested as the reason that the feeding value (higher performance and improved efficiency) of the distillery co-product has been observed in several trials to be higher than equal amounts of the original grain (Klopfenstein, 1996; Klopfenstein and Grant, 2001).

The fiber in DDGS from corn results in >40% neutral detergent fiber (NDF), which is the sum of cellulose, hemicellulose and lignin. While particle size is too small for the NDF in DDGS to provide the physiological effects needed by cattle from fiber, over 60% of the NDF is in the form of hemicellulose, and therefore nutritionally useful.

Wet distillers grains

Cost of transport and rapid spoilage make use of wet distillers grain practical only where large concentrations of cattle are relatively near an ethanol plant, but where such opportunities exist, the reduced energy requirements for drying make the product attractive for both buyer and seller.

Apart from moisture content, the nutrient content of DDGS and wet distillers grains are very similar, provided that the same amounts of solubles are added. Interestingly, some of the studies with growing cattle have suggested an advantage in growth performance of feeding wet *vs* dry distillers grains. Speculation has been that normal drying may reduce energy value to some extent, however this has been difficult to prove (Klopfenstein, 1996; Klopfenstein and Grant, 2001).

A very real concern when feeding wet grains is molding and the associated potential for mycotoxin contamination, which can both reduce performance and cause animal health problems. Addition of preservatives at the alcohol plant may extend the shelf life of wet grains, especially during the summer. Experiments with CakeGuard™ (Alltech Inc.), a preservative based on a mixture of organic acids, have shown that use rates of 2 kg/t prevented mold growth for two weeks when stored at room temperature. In the field, CakeGuard™ is being used with success at 1 kg/ tonne to extend stability of wet cake beyond the typical four days expected.

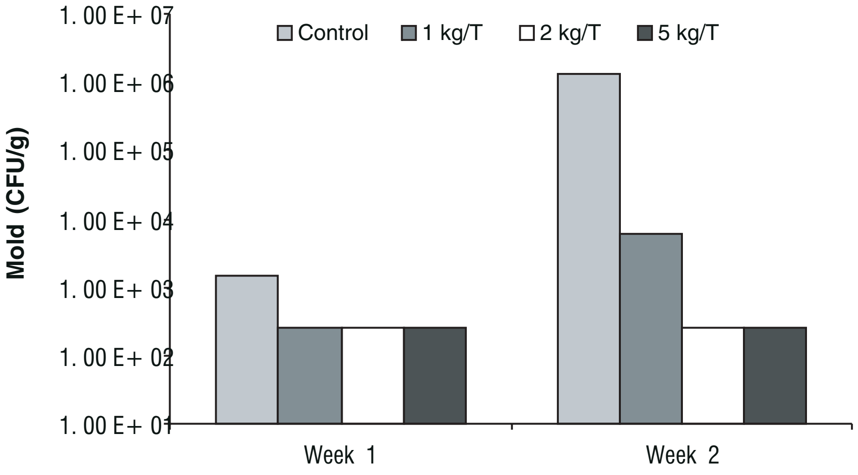


Figure 4. Effect of CakeGuard™ addition rate on mold growth in wet distillers grains weeks 1-5 through storage.

PIGS, POULTRY AND DISTILLERS GRAINS WITH SOLUBLES

The pig sector arguably represents the largest potential for increased use of DDGS. Until recently, very little DDGS has been used in diets fed pigs. The primary reasons include variability in quality and nutrient content among sources, poor amino acid digestibility due to overheating during drying, concerns about the high fiber content, and cost competitiveness with the corn, soybean meal and dicalcium phosphate (inorganic phosphorus source) they replace (Shurson *et al.,* 2003). Research with all classes of pigs at the University of Minnesota and other midwestern universities has demonstrated conclusively that the higher energy, digestible amino acid and available phosphorus content of DDGS from modern ethanol plants allows inclusion at levels in excess of 20% in many diets (Table 7).

Table 7. Use rate recommendations for high quality DDGS in diets for pigs.

Production Phase	*Maximum % of diet*
Nursery pigs (>7 kg)	25
Grow-finish pigs	20
Developing gilts	20
Gestating sows	50
Lactating sows	20
Boars	50

Shurson *et al.*, 2003

The higher levels of inclusion listed in Table 7 assume that diets are formulated based on the digestible amino acid (as opposed to total protein) and available phosphorus content of diet ingredients (Shurson *et al.,* 2003). This is an important point to note in terms of marketing DDGS. Nutritionists, particularly those feeding pigs and poultry, need to know the amounts of digestible amino acids in an ingredient, not simply crude protein content.

DDGS, like corn, is fairly low in the amino acid lysine, which is the first limiting amino acid for growth. Modern ethanol plants, with process controls that help maximize starch extraction, fermentation efficiency, and other upstream operations, produce less variable DDGS with higher levels of more digestible lysine (see Table 2). As processes further improve and quality control of co-products reaches higher standards, an increasing number of pig feed manufacturers will have the confidence in DDGS needed to boost inclusion levels from the currently conservative 5 and 10%.

ENVIRONMENTAL IMPACT

Increasing use of DDGS in pig diets can ultimately have a large and beneficial environmental impact. Owing to the indigestibility of the phosphorus in corn, the phosphorus content of pig manure is a major concern. Phosphorus carried in runoff from land used to spread manure threatens surface waters, causing algae blooms in streams. Regulations limiting the amounts of phosphorus that can be added to a given land area are being put into place in virtually all major agricultural regions, which in turn can limit the number of animals reared and the overall profitability of the farm. Given both the expense of adding inorganic phosphorus to animal feeds and the desire to reduce the environmental impact of manure, the high available phosphorus content of DDGS has much to offer pig feed manufacturers.

POULTRY

Use of DDGS in chickens and turkeys has historically been limited, however its value in providing 'unidentified growth factors' that influence hatchability and efficiency have long been recognized. While the fiber content of DDGS limits its use to a greater extent in poultry than in other species, research with the product from modern plants has shown that when high quality DDGS is used in diets formulated on an available amino acid basis, performance and economic advantages are the result. Work demonstrating these responses in turkeys has resulted in an increasing amount of DDGS being included in turkey diets in Minnesota.

Contaminants: mycotoxins and antibiotics

A quality concern for use of high levels of distillery co-products is the threat of mycotoxin contamination. Mycotoxins present in the grain mashed for fermentation are not destroyed during fermentation and distillation, since heat stability varies among toxins. In addition, moisture conditions in storage are critical for prevention of surface mold. As long as DDGS is dry to <15% moisture and is cool entering storage, the potential for mold growth is no greater than in other feed ingredients. It should be noted, however, that establishing procedures for mold prevention (along with mycotoxin analysis) is something purchasers increasing look for from ingredient suppliers. Commercial feed mixes often include mycotoxin adsorbents as a precaution against mold growth in feed stored at customer farms. One adsorbent is based on a yeast cell wall component modified to maximize the range of toxins adsorbed, which are thereby prevented from harming the animal (Table 8).

In recent years, the use of in-feed antibiotic growth promoters has markedly declined. Both a consumer issue and a potential health concern because it might contribute to resistance of pathogens to similar drugs used in human medicine, feed manufactures are removing such compounds from feeds and 'antibiotic-free' is a marketing claim made for some food animal products as well as animal feeds. Further, the exclusion of all or some antibiotic growth promoters (virginiamycin is a relevant example) is a legal requirement in some arts of the world. The possibility of any measurable antibiotic residue, no matter how small the amount, will increasingly restrict market opportunities for ethanol co-products.

Table 8. Mycotoxin binding efficiency of Mycosorb[1].

	Amount bound[2] *(%)*
Total aflatoxins B1+B2+G1+G2)	85.23
Zearalenone	66.66
Citrinin	18.41
T2-Toxin	33.39
Fumonisin	67.00

[1]Minus values of control group

[2]Alltech Inc.

Replacing animal proteins: opportunities for export?

Good quality vegetable protein sources are in increasing demand across the world. While it can be said that this would be true in any case, one of the changes in animal feeding programs in Europe following BSE was a reduction in use of animal by-product ingredients. Higher prices and lower availability of fish meal have increased use of soybeans as protein sources in poultry feeds in both Europe and South America. While shipping costs, the acceptability of by-products from genetically modified crop varieties and other issues affect export patterns, the trend away from animal by-products may provide opportunities as experience with, and availability of, ethanol co-products increases.

Conclusions

Feed industry professionals that know both the North American food animal market and the nature and advantages of modern distillery co-products have done the math: the expected increase in DDGS could be used in North America, and to great advantage to both ethanol and feed manufacturers. To turn those calculations into reality, however, the gap between the two industries must be bridged with the kinds of information that will give feed manufacturers knowledge and confidence in the feeding value of DDGS. As the sellers, the ethanol industry has the job of promoting and ensuring consistency of DDGS features and benefits. As in selling any product, knowing the target market and how the product fits into it will be critical; and attention to nutritional quality will be needed.

The ruminant sector has the potential to use more distillery co-products, particularly by taking advantage of the opportunity afforded to eliminate starch overload and reduce acidosis. However the biggest increase in DDGS usage can take place in the pig industry where current usage is undeveloped and the research is in place to move forward. Another advantage is that to a large extent, the pigs in the midwestern US are located comparatively close to the ethanol industry. In contrast, the large dairy cattle markets in California, Texas and the southeastern US are farther away. The poultry industry also has the ability to use more DDGS, though inclusion rates are more restricted by DDGS fiber content. However, increasing attention to quality and consistency details that affect amino acid availability will offer poultry feed manufacturers an increasingly attractive ingredient option.

References

Ergul, T., C. Martinez Amezcua, C. Parsons, B. Walters, J. Brannon and S. L. Noll. 2003. Amino acid digestibility in corn distillers dried grains with solubles.

Hancock, J. D. 1995. The value of distillers co-products in swine diets. Proc. Distillers Feed Res. Council. 50:13-20.

Klopfenstein, T. J. 1996. Distillers grains as an energy source and effect of drying on protein availability. Anim. Feed Sci. Technol. 60:201-207.

Klopfenstein, T. and R. Grant. 2001. Uses of corn coproducts in beef and dairy rations. Presented at the Minnesota Corn Growers Association Technical Symposium, Bloomington, MN.

Knott, J., J. Shursonand J. Goihl. 2001. Effects of the nutrient variability of distiller's solubles and grains within ethanol plants and the amount of distiller's solubles blended with distiller's grains on fat, protein and phosphorus content of DDGS. University of Minnesota DDGS Website: *http://www.ddgs.umn.edu/research-quality.htm*

Markham, S. 2003. DDGS production. Presented at the NCGA Regional Distillers Grains Workshop, Des Moines, IA. *http://www.ddgs.umn.edu/ppt-pqd.htm*

NRC. 1998. Nutrient Requirements of Swine. 10th revised edition. National Academy Press, Washington, DC.

Schingoethe, D.J. 2001. Using distillers grains in the dairy ration. National Corn Growers Association Ethanol Co-products Workshop. Nov. 7, Lincoln, NE.

Shurson, J., M. Spiehs, J. Wilson and M. Whitney. 2003. Value and use of 'new generation' distiller's dried grains with solubles in swine diets. In: Nutritional Technology in the Feed and Food Industries, Proceedings of the 18th Annual Symposium (TP Lyons and KA Jacques, eds). Nottingham University Press, UK. 223-235.

Chapter 28

Biorefineries: the versatile fermentation plants of the future

KARL A. DAWSON

North American Biosciences Center, Alltech Inc., Nicholasville, Kentucky, USA

Introduction

Traditional ethanol production systems are designed to efficiently produce alcohol from simple sugars derived from grains and products from sugar processing. In their natural form, these sugars are in the form of polysaccharides that must be subjected to degradative processing before they can be subjected to the metabolic activities of microorganisms during active fermentation. Since polysaccharides are generally derived from complex plant materials, no single processing step or enzyme treatment will release all of the sugars. As a result, the conversion of plant material to useful sugars is generally incomplete, and a large proportion of the material initially entering the ethanol processes is not converted to ethanol and remains unfermented. One of the key challenges for the modern alcohol industry is defining systems for economically converting these unfermented materials into useful or value-added co-products that can contribute to the profitability of the fermentation plant. This chapter will describe some concepts that can be used to extend the bioprocessing capabilities and flexibility of alcohol production plants to make them more sustainable and economically viable.

The biorefinery concept

In the past, processes in traditional alcohol distilleries have been optimized for the efficient production of a single product, ethanol. The value of the plant was largely defined on the basis of its ability to efficiently produce alcohol. There was little consideration given to the 'by-products' that are produced, even though these materials can account for more than a third of the organic materials entering the plant. The markets for these materials have often been considered secondary to that of ethanol produced. With increased emphasis on economic accountability and the need for controlling waste streams and environmental impacts, it is now becoming critical to update some of the basic processes used to produce ethanol. Advances in biotechnology make these updates particularly timely, since our new understanding of biochemical systems and specific microorganisms has provided us with many new tools that can be used to upgrade the basic processing systems used in ethanol production.

The term 'biorefinery' has been used to describe chemical production facilities that use biological systems (microbial fermentations and enzymatic conversions) to efficiently catalyze key chemical transformations (Abbas and Cheryan, 2002). As we have moved into the age of molecular biology and metabolic engineering, we now see that innovative applications of biology can have a tremendous impact on both the economic and the practical engineering of the ethanol production process. In broader terms, biorefineries can be seen as very versatile facilities that are not bound to the production of an individual product, but can instead use a variety of substrates and many different processes to produce a wide range of products with minimum amounts of waste (Figure 1). Instead of thinking of traditional processes that produce only one or two products from a single substrate (Figure 2a), it is important to think about the value of systems that produce many different products (Figure 2b) from a variety of

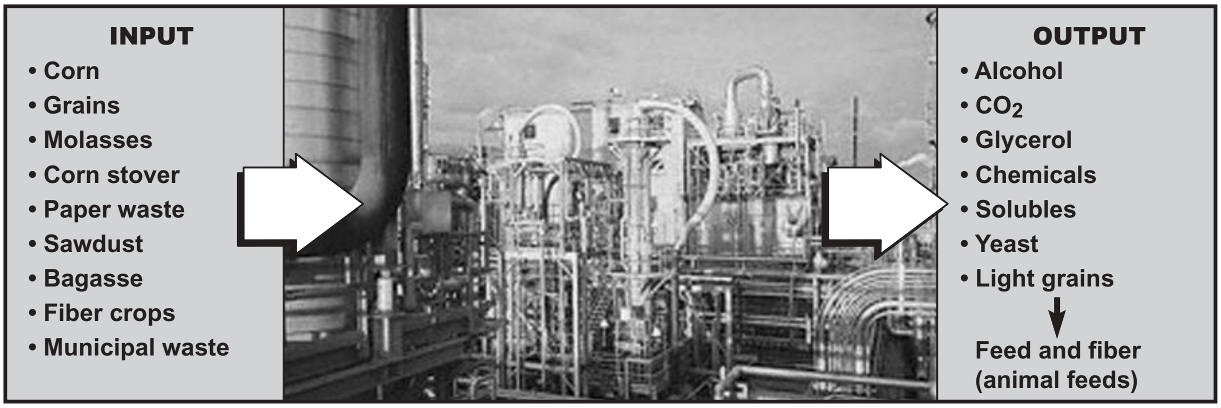

INPUT
• Corn
• Grains
• Molasses
• Corn stover
• Paper waste
• Sawdust
• Bagasse
• Fiber crops
• Municipal waste
OUTPUT
• Alcohol
• CO2
• Glycerol
• Chemicals
• Solubles
• Yeast
• Light grains
Feed and fiber (animal feeds)

Figure 1. A biorefinery.

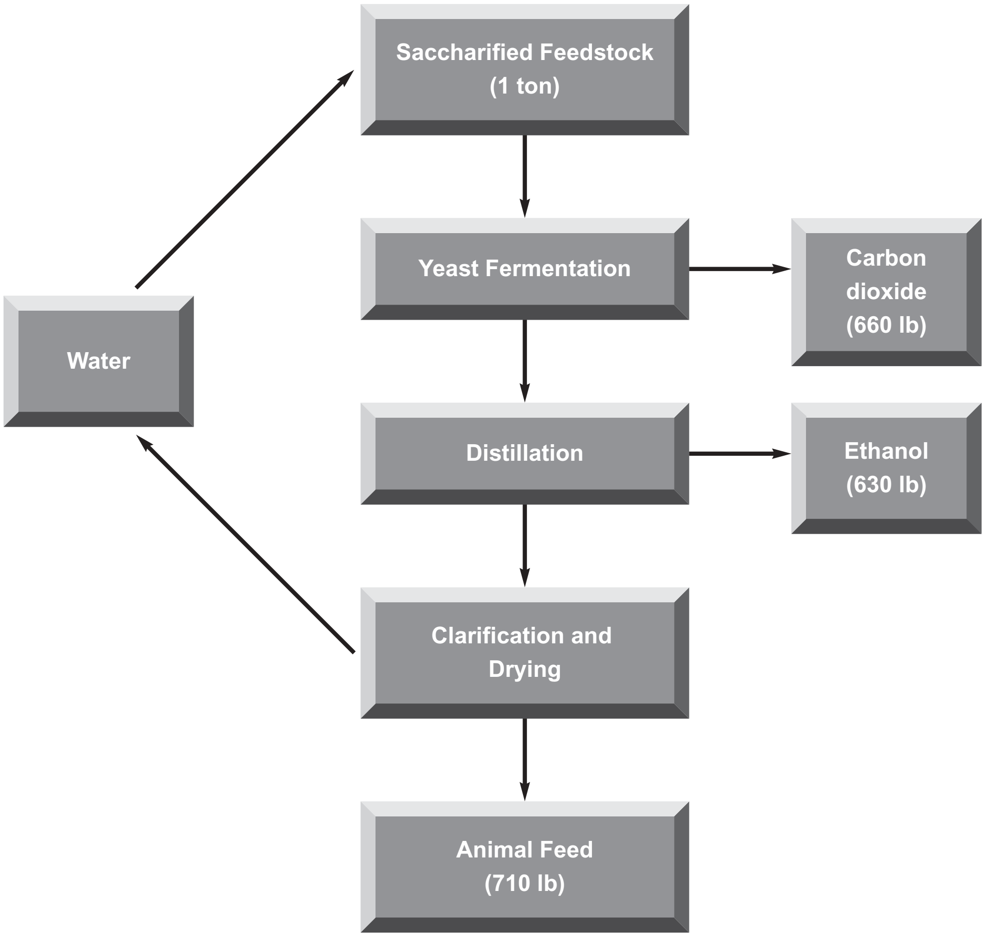

Saccharified Feedstock (1 ton)
Yeast Fermentation
Carbon dioxide (660 lb)
Water
Distillation
Ethanol (630 lb)
Clarification and Drying
Animal Feed (710 lb)

Figure 2a. A bioprocessing system that focuses on the efficient production of ethanol.

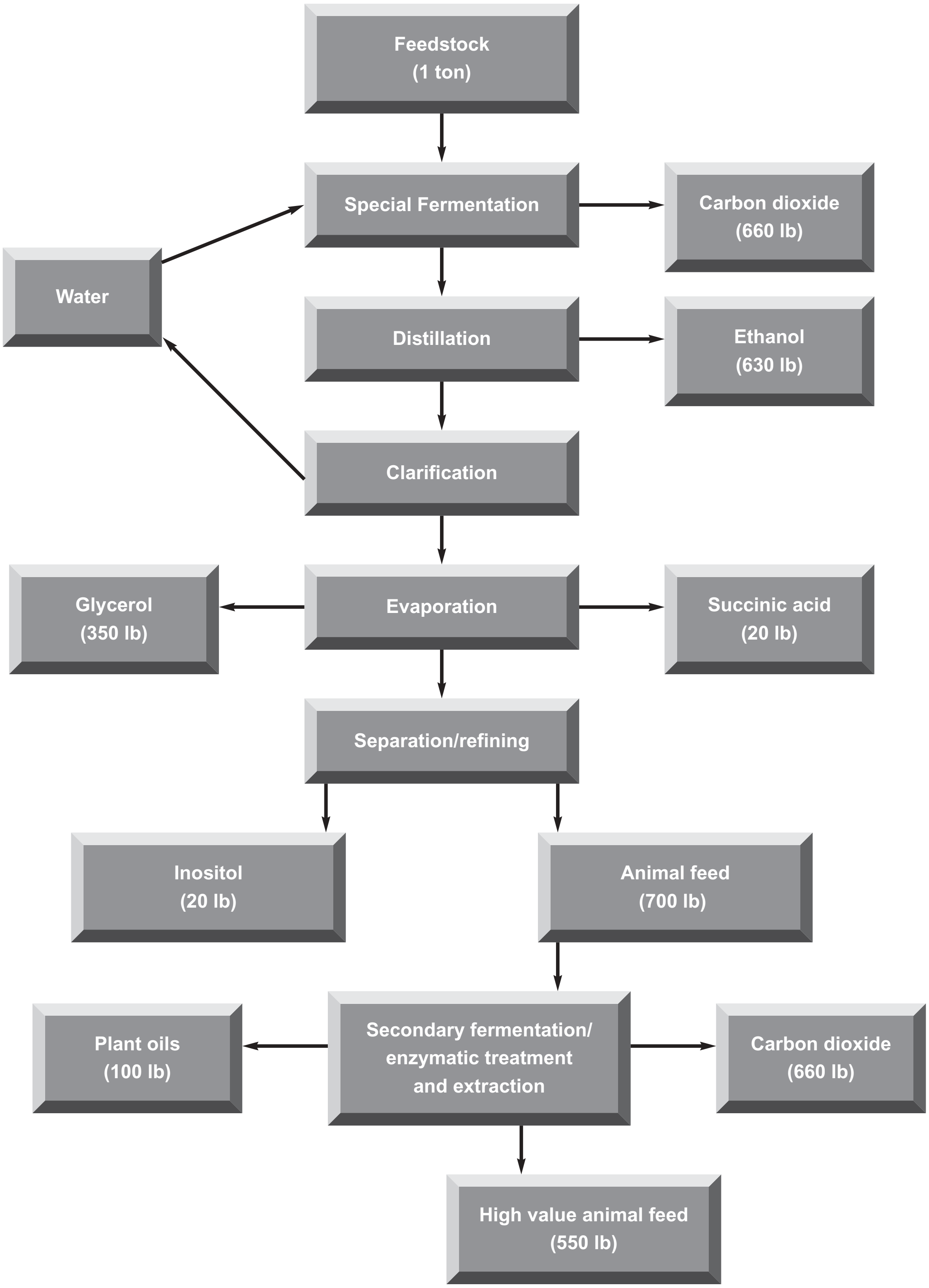

Feedstock
(1 ton)
Special Fermentation
Carbon dioxide
(660 lb)
Water
Distillation
Ethanol
(630 lb)
Clarification
Glycerol
(350 lb)
Evaporation
Succinic acid
(20 lb)
Separation/refining
Inositol
(20 lb)
Animal feed
(700 lb)
Plant oils
(100 lb)
Secondary fermentation/
enzymatic treatment
and extraction
Carbon dioxide
(660 lb)
High value animal feed
(550 lb)

Figure 2b. A bioprocessing system that focuses on production of a range of products with minimal waste.

substrates. In some cases, this may be at the expense of ethanol production. Ideally, the economic value for the biorefinery would come from its versatility. Biorefineries that can use a wide variety of feedstocks and produce a variety of co-products will have the ability to adapt their processes to address external economic pressures.

In addition, there are a number of other basic pressures that make the biorefinery concept attractive for modern ethanol production systems. In particular, environmental issues and the need for controlling waste streams from chemical production systems are becoming the driving force for changes in ethanol production. In this respect, it is becoming possible to use biotechnology to design biorefineries in a way that will allow them to be emission-free, efficient in their use of energy and water, and yet flexible enough to profitably provide useful products from inexpensive substrates.

Feedstocks for the biorefinery

Most modern ethanol production facilities rely on starch or sucrose from grains and sugar cane as the basic substrate for ethanol production. The sugars in these materials are readily released from polysaccharides for fermentation by different combinations of heat and enzymatic treatment. However, the lignocellulose in plant fiber or biomass is now being seriously considered as a feedstock for alcohol production (Lynd *et al.*, 1991). This material is the most abundant source of carbohydrate in the world, but is much more difficult to degrade than the starches derived from grains. The use of this fibrous biomass in a biorefinery provides mechanisms for using renewable and sustainable substrates as a readily adaptable energy source or as a precursor for numerous foods and chemicals (Gong *et al.*, 1999). While major advances have been made in converting lignocellulolosic substrates to more valuable materials, there are a number of basic limitations that have prevented the adaptation of such technologies to large-scale production of vendable products like ethanol. One major challenge with fibrous substrates has been the lack of efficient biochemical mechanisms for preparing the sugars for fermentation. In recent years, there has been considerable interest in the development of specific enzyme systems for producing fermentable substrates from fibrous plant materials and waste products. Special projects have focused on the development of inexpensive cellulase and xylanase enzyme systems with improved catalytic activities. Major development efforts using both fiber-digesting enzymes and new thermochemical processes have allowed for the saccharification of non-traditional lignocellulosic substrates (Kaylen *et al.*, 2000). However, there is also concern about the use of the pentose sugar derived from the hemicellulose fraction of fibrous plant material because these sugars are not used by *Saccharomyces cerevisiae* in traditional ethanol production systems. Feedstocks may also include waste products from the sugar and starch refining industry, milk sugars in whey, corn fiber, forest products and even municipal and food industry wastes. Innovative applications of biotechnology and engineering principles are needed to obtain maximum benefit from this array of substrates.

Traditional co-products from ethanol production processes

Traditionally, co-products derived from ethanol production processes are those that can be obtained by simple physical or chemical extractions associated with grain processing. In many cases, these products have their own intrinsic value as products for human and animal feeds or as feedstocks for other chemical production processes (Table 1). The value of these materials is often determined by the price of competitive products coming from the petroleum industry.

Probably the most important co-products from the alcohol production process are the **distillers dried grains** (DDG) and **distillers dried grains with solubles** (DDGS). This material is the residue remaining after grain processing, fermentation and the distillation of the alcohol. As a result, the composition of these materials is based on biological-based transformations that are brought about by enzyme hydrolysis of the substrate and those associated with the yeast during the fermentation process. Applications of these co-products often take advantage of the

Table 1. Some traditional co-products from various ethanol feedstocks[a].

Feedstock	*Product*	*Uses*
Wheat	Gluten (proteins)	Human foods, building materials, and pharmaceuticals
	Bran (fiber)	Cereal-based food products
	Germ	Fortification of bakery products
	Distillers grains	Animal feeds, human food ingredients
Corn	Zein (protein)	Water resistant films and fibers
	Corn fiber	Animal feeds
	Corn germ	Protein and mineral supplements for baked goods
	Gluten meal	Protein supplement
	Corn oil	Source of gums, lecithin, emulsifiers and antioxidants. Used extensively in human foods
	Distillers grains	Animal feeds
	Stillage (or steep liquor)	Source of nutrients for industrial fermentations
Oats	Proteins	Protein fortification of human foods
	Oat starch	Cosmetic production
	Oat hulls	Production of adhesives and abrasive filters
Barley	Protein	Protein for human food and animal feeds
	Barley fiber	Animal feeds and human foods
	Enzymes	Industrial applications (distilling industry)
	Distillers grains	Animal feeds
	Tocopherols	Antioxidant for food products

[a]Adapted from Torre, 1999.

high fiber and high protein content that remains after the preparation of the readily fermentable sugars and starch for fermentation. Distillers grains account for the largest single co-product from the ethanol industry in the United States. Close to 10 million tons of these DDG is produced in the United States each year. Because of the high fiber content, many of these co-products are typically used as feeds for ruminants (beef and dairy cattle). However, many of them also contain useful substrates that could provide a potential starting point for further biotransformation into high value products. Such transformations provide higher value feeds for other livestock production systems.

Changing the value of traditional co-products in a biorefinery

Distillers grains are considered to be the standard co-products of the alcohol fermentation process in dry mill, ethanol production systems, but can be quite variable in quality. While there has been some attempt to differentiate distillers grains obtained from different sources, most of these materials are currently sold as commodities into the animal feed market. Probably the best opportunity for developing the biorefinery concept is related to the innovative use of the distillers grains. Since distillers grains are most often valued as a protein source for food animals, strategies that increase the protein content, alter the availability the protein, and improve amino acid composition may have considerable value. Such strategies may also improve the profile of the key nutrients for animal production and allow for a more economical application of these spent grains in monogastric animals (pigs) and poultry diets. A number of approaches are currently available for modifying distillers grains and improving their overall value as co-products. These can be simple supplementation strategies, but many of them are true biotransformations that could be carried out in a biorefinery setting.

One approach for improving the value of distillers grains would be to develop supplementation strategies that improve nutrient profiles. This can be accomplished by adding deficient or unique amino acids, vitamins, and minerals in a way that complements the basic nutritional value of spent grains and other dietary components. Since the results of these strategies are often reflected in improved animal performance, it is often difficult to evaluate the economic value of such approaches based on simple nutrient analysis. Instead, it must be based on the improved performance of the livestock (Table 2). Early studies with one such supplementation strategy using proprietary vitamin and mineral mixes have indicated that it is possible to get a three-to-one return on processing costs when such strategies are used to improve milk production in dairy cattle.

Another approach for improving the value of the distillers grain could focus on the relatively poor digestibility of the protein and fiber fractions in the spent grains. Current estimates of the availability of the most limiting amino acids in distillers grains is estimated to be about 65% in swine diets (Dale and Batal, 2002). This suggests that a large portion of the protein ingested by the pig is not subject to digestion and is unavailable as a nutrient. A biotransformation with an enzymatic treatment that increases the amino acid availability from 65 to 90% could significantly alter the value of spent grains. Currently, biotechnology may provide specific enzyme complexes that can bring about the required improvements in amino acid availability. If such modified grains are formulated into a diet for a pig in place of soybean meal, savings in the cost of a ton of feed can be demonstrated (Table 2). In this case, biorefinery steps that allow for the pre-treatment of distillers grains with an enzyme may increase the basic economic value of the distillers grain as a feed ingredient in pig diets by as much as 20 to 30%.

Probably the most effective way to enhance the value of distillers grains would be to expose them to a more complex biotransformation using a secondary fermentation system. This process could use selected strains of fungi and bacteria in combination with a short fermentation period to further alter the grain composition. This process could increase the protein content, increase the lysine content of the protein, and improve the overall digestibility of the protein. This fermentation process may also decrease the fiber content of the spent grains and increase their overall energy availability. Such manipulation could provide a much more balanced protein to the animal in the form of a highly digestible feed ingredient. Simple fermentations that increase protein content from around 30% to up to 45%, increase amino acid availability from 65 to 90%, and improve lysine content (from 1.35% to 2.7%) can improve the value of the spent grains as a feed ingredient. This can result in up to a 60% increase in the basic economic value of the distillers grains as a feed supplement (Table 2). These examples clearly show the value of strategic applications of microbial induced biotransformations in a biorefinery setting.

Products from the biotransformation processes

Commercially, a number of products are

Table 2. Potential value of developing fermentation co-products from spent distillers grains for swine.

Strategy	*Goal of strategy*	*Decrease in feed cost relative to soybean meal*	*Added value per ton of distillers grains*[a]
Strategic supplementation with nutrients or dietary adjuncts (minerals, vitamins, amino acids, or enzymes)	Improved or more balanced nutrient profile	Depends on cost of supplement	Value only obtained through enhanced performance of pigs 1:3 return on investment has been reported
Enzymatic treatment (hydrolytic pre-digestion)	Improved digestibility or improved nutrient availability	$2.00	Approx. $20.00
Secondary fermentation (microbial treatment or ensiling)	Improved nutrient content; improved nutrient profile; improved nutrient digestibility and availability	$6.00	Approx. $60.00

[a] Changes in the value of distillers grains when formulated relative to soybean meal in swine diets

produced using the basic metabolic activities of living organisms (Table 3). Many of these products are produced in well-defined fermentation systems that convert low quality substrates into high value, vendable products or chemical precursors for other manufacturing processes. Alternatively, some of these biotransformations can be achieved by applying specific enzyme systems. By applying new fermentation processes, biotechnology can provide tools that can be used for the production of new inexpensive enzyme systems for more cost-effective biotransformations. Innovative applications of some of the fermentation processes or enzyme treatments into integrated production systems will be important to the development of flexible biorefineries (Figure 2b).

Table 3. Some industrial products currently produced using fermentation technologies that might be produced in a biorefinery using microbial biocatalysts.

Ethanol
Methanol
Citric acid
Glycerol
Xylitol and other sweeteners
Astaxanthin
Lactic acid
Polylactide polymers
Emulsifiers and surfactants
Vitamins (riboflavin)
Fatty acids and oils
Enzymes
Specific pharmaceuticals and specific heterologous proteins
Single cell protein

Microorganism use in biotransformations

The processing power in modern biorefineries is often limited by the metabolic power of microorganisms or biocatalysts available. Modern biotechnology is now capable of engineering new metabolic systems that increase the flexibility of biorefineries. These are based on both traditional or naturally occurring microorganisms and genetically modified organisms. Microorganisms used for biotransformation fall in to three major groups: classical yeast, fungi, and bacteria. Many types of organisms have been examined for their ability to contribute to bioconversion of basic feedstocks and metabolic intermediates within biorefineries.

YEAST

While the metabolic activities of many different types of microorganisms have been examined for functional roles in biorefinery systems, currently the vast majority of commercial fermentations depend on active yeast cells. These organisms are the basic biocatalysts that are central to the modern fermentation industry. The most important yeasts in this respect are from the species *Saccharomyces cerevisiae.* There are several thousand strains of *S. cerevisiae* available to the fermentation industry. They can be differentiated biochemically and by their basic fermentation activities. However, a number of non-conventional yeast have been tested and used as biocatalysts for the production of specialized fermentation products (Table 4). Representative strains from these different yeast species carry out a variety of biochemical processes that make them attractive for driving fermentation and biotransformation processes in an integrated biorefinery setting.

Metabolic engineering is a concept that uses powerful genetic engineering approaches and microbial physiology in strain development strategies for improving the biocatalytic capabilities of microorganisms (Olsson and Nielsen, 2000). Metabolic engineering of yeast now allows for the development of fermentation processes that integrate much of our knowledge about physiological, metabolic, and biochemical properties of this group of organisms using specific genetic engineering technology to improve specific bioprocesses. This is becoming a very successful way of changing our traditional fermentation technologies. For example, recently, genetic modification techniques have made a variety of yeast strains available that have very specific metabolic activities (Gong *et al.*, 1999, Mouiruzzaman *et al.*, 1997). These molecular tools are extremely powerful and make it possible to initiate metabolic engineering programs that allow for new processing strategies. Genetic modification has been especially important in developing strains of yeast that can use the xylose derived from

Table 4. Some non-conventional yeast uses available for use in modern biorefineries.

Yeast	*Applications*
Candida boidinii	Vendable chemicals and enzymes production from fatty acids, methanol and ethanol
Candida butyrii	Ethanol production from xylose
Candida guilliermondii	Xylitol and citric acid production from pentoses and cellulose
Candida shehatae	Ethanol production from xylose
Candida tenuis	Ethanol production from xylose
Candida tropicalis	Xylitol and other vendable chemicals produced from pentoses and petroleum base substrates
Candida utilis	Wide variety of organic chemicals from fibrous substrates
Cryptococcus laurentii	Enzyme and organic acid production from waste oils
Debaryomyces hansenii	Xylitol and citric acid production from xylose and hydrocarbons
Geotrichum spp.	Enzyme production
Hansenula polymorpha	Protein production from methanol
Kluyveromyces fragilis	Thermotolerant ethanol production from fiber hydrolysates
Kluyveromyces lactis	Ethanol, enzyme and heterologous protein production from whey
Kluyveromyces marxianus	Ethanol production from whey, xylose, and pectin
Pachysolen tannophilus	Ethanol and xylitol production from xylose from pentoses
Phaffia rhodozyma	Carotenoid pigment production from corn by-products and wood hydrolysates
Pichia pastoris	Heterologous protein and enzyme production from methanol and oxygenated hydrocarbons
Pichia stipitis	Ethanol production from xylose and xylans (hemicellulose hydrolysates)
Schizosaccharomyces pombe	Ethanol production from simple sugars
Schwanniomyces occidentalis	Halophilic biotranformations of starch to organic chemicals
Yarrowia lipolytica	Citric acid production from plant oils, hydrocarbons, and petroleum products

lignocellulose digestion (Ho *et al.*, 1999, Krishnan *et al.*, 1999) and the development of *S. cerevisiae* strains that contain ß-glucosidase and directly produce ethanol from cellulose (Cho and Yoo, 1999). When combined with our understanding of molecular genetics, our sophisticated understanding of yeasts and their physiological activities make them attractive as biocatalysts in biorefineries.

FUNGI

The metabolic flexibility and unique substrate range of fungi make them potential contributors to the modern biorefinery. However, relative to the yeast, very few species have been evaluated for their contributions to commercial production systems (Table 5). These organisms can grow on a variety of substrates and are readily manipulated to produce a variety of useful end products. Fungi are often used to economically produce industrial enzymes. This enzyme production often takes advantage of the ability of these organisms to grow on solid substrates, such as wheat bran, with relatively low moisture contents (around 50%). This characteristic makes it possible to grow these organisms in surface culture systems (solid state fermentation) with exceptionally high yields of enzyme. This has been the basis for developing large scale 'Koji' or solid state fermentation systems (SSF). Enzyme complexes produced in a SSF system have a number of unique characteristics, including a broad range of side or auxiliary activities, that make them more powerful than purified enzymes obtained from traditional liquid fermentation systems. These types of enzyme

Table 5. Some fungi that may contribute to biorefinery processes.

Fungal group	*Recognized functions*
Aspergillus niger	Citric acid and enzyme production from a variety of substrates
Penicillium spp.	Enzyme and antimicrobial production
Rhizopus oligospora	Enzyme production from a variety of substrates
Trichoderma reesei	Enzyme production from fibrous substrates

systems could be attractive co-products in a biorefinery system where partially dried distillers grains are available as a substrate. For example, it is possible that DDGS could be used as a substrate for producing an enzyme such as Rhizozyme™, which would make it unique not only in ability to totally replace other saccharifying enzymes (saving \$0.04-\$0.05 gallon), but bring about an increase in yield since hemicellulose/cellulose would be degraded.

It is also possible to think about metabolic engineering of fungal cultures to make them more useful as tools in a modern biorefinery. While mutant strains of fungi have been used for many years to produce commercial enzymes, systems for routinely manipulating their genetic makeup are still lacking. New frontiers will open up when reliable technologies for metabolic engineering of fungi become readily available.

BACTERIA

Bacteria have a long history of contributing to commercial fermentation processes and are often considered in the development of unique processes in biorefinery systems. The ability of bacteria to use multiple substrates and their susceptibility to genetic manipulation make them attractive candidates for biocatalytic roles. However, bacterial fermentations in commercial settings generally tend to be less robust than those associated with yeast and fungi. As a result, care must be taken in preparing substrates and facilities that will support bacterial fermentations. Many types of naturally-occurring bacteria have been examined for their potential role in commercial fermentation systems (Table 6). Such organisms can provide a wide variety of biochemical activities that may be integrated into unique fermentations systems in a biorefinery.

Molecular manipulation and genetic engineering have also allowed for the development of many new strains of bacteria with unique fermentation capability. For example, strains of bacteria that contain active lignocellulose-degrading enzymes have been developed and can be used to provide innovative microbial activities in biorefineries. Genetic manipulation of *Escherichia coli* provides one of the most powerful tools for modern biotechnology and provides the best studied system for manipulating microbial processes. Manipulated strains of this organism are commonly used to produce many types of pharmaceutical products, enzymes, and diagnostic reagents. Specific strains of this *E. coli* that produce ethanol have also been developed (Ingram *et al.*, 1999). Some of these strains can use a variety of substrates, including some of the pentose sugar derived from the degradation of the hemicellulose fraction of plant fiber (Dien *et al.*, 1999).

In recent years, there has been considerable interest in using heat resistant thermophilic bacteria in industrial fermentation processes (Scopes, 1997). Many of these bacteria naturally produce mixtures of ethanol and organic acids, but tend to be less ethanol tolerant than yeast. These organisms are uniquely adapted to carry out fermentation at temperatures higher than 50ºC. Application of these types of organisms may allow for an increase in the temperature range for biocatalytic activities and allow for high temperature strategies in biorefineries.

Table 6. Some bacteria that may have applications in modern biorefineries.

Bacterial group	*Application*
Arthrobacter spp.	Enzyme production
Bacillus spp.	Enzyme production
Clostridium spp.	Acetone and butanol production
Erwinia spp.	Ethanol and organic acid production from hexoses and pentoses
Escherichia coli	Ethanol and organic acid production from hexoses and pentoses
Klebsiella oxytoca	Ethanol and organic acid production from hexoses and pentoses
Lactobacillus spp	Used in a wide variety of food products
Pseudomonas spp.	Enzyme production and complex biotransformations
Streptomyces spp.	Organic chemical, antimicrobial and enzyme production
Thermoanaerobacter spp.	Thermophilic ethanol production
Zymomonas mobilis	Ethanol from fiber and starch hydrolysates

Other tools from biotechnology

In addition to allowing for the development of new strains of microorganisms, molecular biology and metabolic engineering techniques are also beginning to provide other new systems and processes that can be used for controlling chemical processes in biorefineries. Specifically, new enzymes and enzyme expression systems have been strategically designed for specific metabolic transformations and are currently being produced and evaluated in a number of applications. Much of this work is focusing on the development of cellulases and xylanases that will allow for more efficient conversion of fibrous biomass into fermentable sugars. Current research programs in these areas not only look at increasing the yields of enzymes, but also at the production of more efficient enzyme systems. Tools such as proteomic analysis, molecular flux analysis, genomic analysis and directed molecular evolution will be important in developing the next generation of enzymes for use in biorefineries (Bon and Picataggio, 2002). The development of such enzyme-based sugar production platforms has been a key part of the strategies for economically converting renewable fibrous biomass to ethanol. It can be expected that similar techniques can be used to develop other enzyme systems to carry out key transformation in biorefineries. The alcohol industry can look forward to the application of many new enzymes and enzyme complexes that will increase the substrate ranges and resistance to some of the environmental extremes associated with industrial fermentations. These will improve the efficiency of ethanol production and improve the value of co-products.

Conclusions

The future of many fermentation plants will depend on their ability to rapidly adapt to changes in the cost of substrates and to obtain the maximum value from the products they produce. The concept of developing flexible biorefinery systems that allow for the use of a variety of feedstocks and that have the ability to produce several high value co-products will be important when determining the value of alcohol production systems. Biotechnology will provide the basic tools needed to develop these integrated biological based processing systems for use in biorefineries.

References

Abbas, C.A. and M. Cheryan. 2002. Emerging biorefinery opportunities. Appl. Biochem. Biotech 98-100:1147.

Bon, E.P.S. and S. Picataggio. 2002. Enzyme and microbial biocatalysts. Appl. Biochem. Biotech. 98-100:163.

Cho, K.M. and Y.J. Yoo. 1999. Novel SSF process for ethanol production from microcrystalline cellulose using the d–integrated recombinant yeast, *Saccharomyces cerevisiae* L2612dGC. J. Microb. Biotech. 9(3):340-345.

Dale, N. and A. Batal. 2002. Ingredient analysis table: 2002 Edition. Feedstuff 74:16-22.

Dien, B.S., L.B. Iten and R.J. Bothast. 1999. Conversion of corn fiber to ethanol by recombinant *E. coli* strain FBR3. J. Indust. Microb. Biotech. 22:272-581.

Gong, C.S., N.J. Cao, J. Du and G.T. Tsao. 1999. Ethanol production from renewable resources. Adv. Biochem. Engin./Biotech. 65:207-241.

Ho, N.W.Y., Z. Chen, A.P. Brinard and M. Sedlak. 1999. Successful design and development of genetically engineered *Saccharomyces* yeasts for effective cofermentation of glucose and xylose from cellulosic biomass to ethanol. Adv. Biochem. Engin./Bioctech. 65:164-191.

Ingram, L.O., H.C. Aldrich, A.C.C. Borges, T.B. Causey, A. Martinez, F. Morales, A. Saleh, S.A. Underwood, L.P. Yomano, S.W. York, J. Zaldivar and S. Zhou. 1999. Enteric bacterial catalysts for fuel ethanol production. Biotech. Progress 15:855-866.

Kaylen, M., D.L. Van Dyne, Y.S. Choi and M. Blasé. 2000. Economic feasibility of producing ethanol from lignocellulosic feedstocks. Bioresource Tech. 72:19-32.

Krishnan, M.S., N.W.Y. Ho and G.T. Tsao. 1999. Fermentation kinetics of ethanol production from glucose and xylose by recombinant *Saccharomyces* 1400(pLNH33). Appl. Biochem. Biotech. 77-79:373-388.

Lynd, L.R., J.H. Cushman, R.J. Nichols and C.E. Wyman. 1991. Fuel ethanol from cellulosic biomass. Sci. 251:1318-1323.

Mouiruzzaman, M., B.S. Dien, C.D. Skory, Z.D. Chen, R.B. Hespell, N.W.Y. Ho, B.E. Dale and R.J. Bothast. 1997. Fermentation of corn fibre sugars by an engineered xylose utilizing *Saccharomyces* yeast strain. World J. Microb. Biotech. 13:341-346.

Olsson, L. and J. Nielsen. 2000. The role of metabolic engineering in the improvement of *Saccharomyces cerevisiae*: utilization of industrial media. Enzyme Microb. Tech. 26:785-792.

Scopes, R.K. 1997. Ethanol from biomass: The potential use of thermophilic organisms in fermentation. Australasian Biotech. 7:296-299.

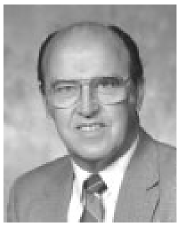
Torre, P. 1999. Co-products from ethanol fermentation: Alternatives for the future. In: *The Alcohol Textbook, 3rd Edition* (K.A. Jacques, T.P. Lyons and D.R. Kelsall, eds). Nottingham University Press, Nottingham, UK, pp. 335-346.

The Alcohol Alphabet

A glossary of terms used in the ethanol-producing industries

Italic type denotes words (or minor variations of words) defined under separate entries.

John Murtaugh
Sept 12, 1936 - Jan 25, 2003



Beginning with his dissertation work on the cooking process through a lifetime of consulting, educating and inspiring others, Dr. John Murtaugh moved the ethanol industry forward. He began this glossary with the 2nd edition of the Alcohol Textbook. Like the rest of John's work, it has an important and very practical purpose. As such, it will be updated and continued in this and successive editions of the book.

A

Å Abbreviation for *Angstrom*.

Absolute ethanol A pharmaceutical term for *anhydrous ethanol*. It is generally defined as having less than 1% water.

Acetaldehyde Otherwise known as ethanal, acetic *aldehyde* or ethylaldehyde. A clear flammable liquid with a characteristic pungent odor. Chemical formula CH_3CHO. Boils at 21°C and freezes at -123.5°C. It is miscible in both ethanol and water. It has a narcotic effect on humans, and large doses may cause death by respiratory paralysis. It is a *congener* in the production of ethanol by *fermentation*, and is usually a major constituent of the *heads* fraction removed in *rectification*.

Acetic acid A colorless liquid with a pungent odor. Flammable at high concentrations. Chemical formula CH_3COOH. Acetic acid may be produced from *ethanol* by *Acetobacter bacteria* under aerobic conditions such as when a completed fermentation is agitated or aerated excessively. Vinegar is a solution of this acid.

Acetobacter A genus of *Gram-negative*, aerobic *bacteria* comprising ellipsoidal to rod-shaped cells as singles, pairs or chains. Otherwise known as acetic acid or vinegar bacteria, they are able to oxidize *ethanol* to acetic acid. They may be responsible for loss of yield in ethanol production if a *fermented mash* is agitated or aerated excessively.

Acetone Otherwise known as 2-propanone, dimethyl ketone or pyroacetic ether. It is a volatile, very-flammable liquid with a characteristic pungent 'mousey' odor and a sweetish taste. Chemical formula CH_3COCH_3. Boils at 56.5°C and freezes at -94°C. It is miscible with water, *ethanol* and most oils. Inhalation may cause headaches, and in large amounts, narcosis. It is a *congener* in the production of ethanol by *fermentation*, particularly from *molasses mashes*. It is also a by-product of the production of *butanol* by *anaerobic* fermentation using the *bacterium Clostridium acetobutylicum*. It tends to concentrate in the *heads* fraction in the *rectification* of *neutral spirit*.

Acid A compound capable of releasing hydrogen ions.

Acidity A quantitative measure of the amount of acid present.

Acid-acid process Term used in *starch* processing when *acid hydrolysis* is used to accomplish both the initial *liquefaction* and the final *saccharification* to simple *sugars*.

Acid-enzyme process Term used in *starch* processing when *acid hydrolysis* is used to accomplish the initial *liquefaction*, and an *enzyme* such as *amyloglucosidase* is used for the *saccharification* to simple *sugars*.

Acid hydrolysis The *hydrolysis* of a *polymer* by the use of acid. In the case of *starch* hydrolysis, acids may be used as an alternative to *enzymes* in either (or both) the *liquefaction* or *saccharification* processes.

Acid washing A process in which *yeast* recovered from a finished *fermentation* is acidified to pH 2.2 for ~20 minutes using an acid like phosphoric acid, to reduce the level of *bacterial contamination* prior to recycling into a new fermentation.

Active dry yeast A yeast preparation made from compressed yeast by careful and controlled removal of moisture to ~5% w/v, such that 10-50 billion yeast/gram remain viable. Storage for a number of months allows plants to eliminate risky propagation ‘in-house’.

Aguardiente An unaged alcoholic beverage produced in Central and South America by the *distillation* of *beer* derived from the *fermentation* of sugarcane juice or *molasses*. It is similar to crude *rum*.

Alcohol A member of a class of *organic* compounds containing carbon, hydrogen and oxygen. Considered to be *hydroxyl* derivatives of *hydrocarbons* produced by the replacement of one or more hydrogen atoms by one or more *hydroxyl* (OH) groups. Under the International Union of Pure and Applied Chemistry (IUPAC) naming system, the name given to an alcohol is derived from the parent hydrocarbon with the final ‘e’ changed to ‘ol’. Thus *methane-methanol*, *ethane-ethanol,* etc. The principal alcohol in fuel and beverage use is *ethanol* (otherwise known as *ethyl alcohol*). The lower molecular weight alcohol (methanol) and higher alcohols are more toxic.

Alcohol Fuel Permit (AFP) A permit issued by the *Bureau of Alcohol, Tobacco and Firearms* allowing the holder to engage in the production of *ethanol* solely for fuel use.

Aldehyde A member of a class of *organic* compounds considered to be derived by the removal of hydrogen atoms from an *alcohol*. Aldehydes tend to be produced as *congeners*, or *by-products* of *fermentation*; and having a lower *boiling point* than *ethanol*, they tend to vaporize more readily and may accumulate as a *‘heads’* fraction in the *distillation* process.

Alkali A compound capable of neutralizing hydrogen ions, usually by generating hydroxyl ions which combine with hydrogen ions to form water.

Alkalinity Specifically in water, a measure of the total bicarbonate and carbonate content, determined by titration to an endpoint using various indicators such as phenylphthalein, methyl orange, etc.

Alpha-amylase (α-amylase) An *enzyme* used in the *liquefaction* of *starch* in the grain *mashing* process prior to *saccharification* and *fermentation*. α-amylase *hydrolyzes* the long-chain *starch molecules* into short-chain *dextrins*. These are more suitable for subsequent saccharification by other enzymes to fermentable *glucose*. (α-amylase is an *endoenzyme* in that it works from the inside of the *amylose molecule).* In beverage alcohol production α-amylase may be derived from *malt* (sprouted barley), but in *fuel ethanol* production the enzyme is obtained solely as a *bacterial* product. The enzyme *molecule* contains a calcium atom, which is essential for its activity.

American Society for Testing and Materials (ASTM) A scientific and technical organization with headquarters in Philadelphia, PA established for 'the development of standards on characteristics and performance of materials, products and services and the promotion of related knowledge'. It sets voluntary consensus standards through committees representing producers and users. ASTM has published standards for *fuel ethanol* and *gasoline* that have been adopted by many states.

Amyl alcohol The principal constituent of *fusel oil*. Otherwise known as *pentanol*. Chemical formula $C_5H_{11}OH$. Eight *isomers* exist, the most common being primary isoamyl alcohol.

Amylase The name given to any *enzyme* that *hydrolyzes* (or breaks down) *amylose*, which is a major component of *starch*.

Amyloglucosidase An *enzyme*, also known as *glucoamylase*, which *hydrolyzes* amylose into its constituent *glucose* units. It is an *exoenzyme* in that it works from an outer end of the *starch molecule*. It is normally used in conjunction with *α-amylase* for the *liquefaction* and *saccharification* of *starch* in the grain *mashing* process, prior to *fermentation*. Amyloglucosidase is a product of fungal growth produced either in liquid fermentation or surface culture fermentation (See Rhizozyme™).

Amylopectin A major component of *starch* (together with *amylose*). The *molecule* is composed of large, branched chains of thousands of *glucose* units.

Amylose A major component of *starch* (together with *amylopectin*). The amylose *molecule* is composed of straight chains of hundreds of *glucose* units. In the grain-*mashing* process for *ethanol* production, amylose may first be broken down into short-chain *dextrins* by *α-amylase*, which are in turn broken down into single *glucose* units by *amyloglucosidase*.

Anaerobic Literally means 'without air'. The opposite of aerobic. For example, *yeast propagation* (or multiplication) is more rapid with aeration (i.e., under aerobic conditions), while yeast produces *ethanol* under anaerobic conditions of *fermentation*.

Anaerobic digestion Process of breaking down waste materials by *anaerobic bacterial* degradation. Normally accompanied by the production of *methane* gas.

Angstrom (Å) A unit of length equal to one hundred-millionth (10^{-8}) of a centimeter, used for measuring the diameter of chemical *molecules*. Thus, in *ethanol dehydration*, a *molecular sieve* material with holes 3 Angstroms in diameter may be used to separate water, which has a 2.5 Å diameter, from *ethanol*, which has a 4.5 Å diameter.

Anhydrous Literally means 'without water'. The term used for a substance that does not contain water. *Ethanol* for fuel use is commonly referred to as anhydrous, because it has had almost all of the water removed. See *absolute ethanol*.

Antibiotic A chemical substance produced by *microorganisms* that kills or inhibits growth of other organisms. Both single antibiotics and combinations (See Lactocide™) are used to control bacterial contamination in the fermentor.

Antifoam (or defoamer) A preparation composed of substances such as silicones, *organic* phosphates and *alcohols* that inhibits formation of bubbles in a liquid during agitation by reducing the surface tension. Antifoam may be used in *ethanol* production to control the development of foam in *fermentors*. Antifoam may also be used to control foam in *beer distillation* to increase the stripping capacity of a *column*.

Antiscalant (or scale inhibitor) A chemical compound or mixture of compounds added to water or *molasses beer* to reduce the incidence of scaling in *heat exchangers* or *distillation columns*. Usually, the principal ingredients of antiscalants are chelating compounds.

Arabinose A *pentose sugar* comprising a major constituent of *hemicellulose*. Chemical formula $C_5H_{10}O_5$. It is not fermented by normal *strains* of distillers *yeasts*. Also known as pectin sugar or gum sugar.

Atomic weight The relative mass of an atom based on a scale in which a specific carbon atom (carbon 12) is assigned a mass value of 12. Also known as relative atomic mass.

Azeotrope The term used to describe a constant boiling mixture. It is a mixture of two (or more) components with a lower *boiling point* than either component alone. For example water, which boils at 100°C, and *anhydrous ethanol*, which boils at 78.5°C, form a constant boiling mixture (azeotrope) at 78.15°C. The *vapor* of the mixture has the same composition as the liquid and therefore no further concentration can be achieved by normal *distillation*. Under normal pressures it contains approximately 97% by volume *ethanol* (194^0 *proof*). It is very expensive in terms of energy to attempt to reach 194^0 proof, so 190^0 proof is generally considered to be the practical, economic azeotrope limit for *fuel ethanol* distillation.

Azeotropic distillation A *distillation* process in which a liquid compound (*entrainer*) is added to the mixture to be separated to form an *azeotrope* with one or more of the components. Normally, the entrainer selected is easily separated from the component to be removed. For example, when *benzene* is used in azeotropic distillation to dehydrate *ethanol*, the *overhead condensate phase-separates* to yield a water-rich layer that can be withdrawn and a benzene-*ethanol* layer, which is *refluxed*.

B

Backset Recycled *thin stillage*. It may be added to the *cooker* or to the *fermentor* and may serve as a partial source of nutrients. It reduces the water required for *mashing* and reduces the volume of liquid residue to be evaporated. Improperly handled, it may be a major source of *bacterial contamination*, and may contain high levels of chemicals that cause stuck/ sluggish fermentations.

Bacteria Microscopic organisms of the kingdom Monera usually characterized by small size, vigorous biochemical activity and lack of a true nucleus.

Bacterial contamination The condition occurring when undesirable *bacteria* become established in a fermenting *mash* and reduce the *ethanol* yield. The *bacteria* use available *sugars* to produce various compounds (*congeners*), particularly acids, which may inhibit *yeast* activity. In severe situations, bacterial contamination may cause serious economic losses.

Balling (or Brix or Plato) A scale used to measure the *specific gravity* of a liquid in relation to that of a *solution* of *sugar* in water. Each unit on the scale is equivalent to 1% by weight of sugar. Thus a *mash* of 20° Balling has the same specific gravity as a 20% w/w sugar solution. The scale is frequently considered to indicate % dissolved solids in a liquid, although this is only true of solutions of pure sucrose. Traditionally, the term 'Balling' has been used in grain distilleries and breweries, while 'Brix' has been used in sugar mills and *rum* or *molasses* alcohol distilleries. The measurement is accomplished by use of a Balling (or Brix/Plato) *hydrometer*, *refractometer* or more recently density meter.

Barbet time See *Permanganate time*.

Bargeload Generally refers to a river barge with a capacity of 10,000 *barrels* or 420,000 gallons.

Barrel A liquid measure equal to 42 US gallons or 5.6 cubic feet. Or, a wooden container used for the aging and maturation of alcoholic beverages. Barrels used for *whisky* maturation are made of oak wood and have a capacity of about 52 US gallons. Barrels may only be used once for aging *bourbon whisky*, so there is a worldwide trade in used bourbon barrels for aging other alcoholic products such as Scotch whisky and *rum*. A US beer barrel contains 31 US gallons.

Base losses The percentage of *ethanol* lost in the *stillage* at the base of a *beer stripping column* or a *rectifying column*. It is virtually impossible to achieve zero base losses, and it would be wasteful of steam. The base losses are generally monitored and controlled in an optimal range determined by steam costs, etc.

BATF Abbreviation for US Bureau of Alcohol, Tobacco and Firearms.

Batch cooking Cooking a set amount of grain *meal,* water and *backset* (if any) in a single vessel as a discontinuous operation. One or more batch cooks may be used to fill a single *batch fermentor*. Batch cooking is mainly confined to the *beverage alcohol* industry and smaller plants in the *fuel ethanol* industry.

Batch distillation Distilling batches of *beer* in a discontinuous operation. It is not commonly used in *fuel ethanol* production, but is used in the beverage *ethanol* industry particularly for the production of special types of heavily-flavored *distillates*.

Batch fermentation The fermentation of a set amount of *mash* in a single vessel in a discontinuous operation.

Beer The name given to the product of alcohol *fermentation*. In *grain alcohol* production, beer may contain from 9->16% *ethanol* (v/v).

Beer preheater A heat-exchanging device used for heating *beer* before it enters a *beer still*. Usually, it also serves as the first *condenser* for the beer still, so that the *overhead vapors* heat the beer.

Beer still The *distillation* unit used for the initial removal of *ethanol* from finished *beer*. It generally consists of a *stripping section* that extracts the *ethanol* from the beer and a *concentrating or rectifying section*, which normally takes the *ethanol* up to 190^0 proof (95^o *GL*). Beer stills may consist of a single tall *column*, or two or more columns standing side by side, linked by vapor pipes.

Beer stripping column See *Beer still.*

Beer well The holding vessel into which finished *beer* is transferred prior to *distillation*.

Benzene Colorless, flammable, aromatic *hydrocarbon* liquid. Chemical formula C_6H_6. Boils at 80.1^{O}C and freezes at 5.4^{O}C. Used as an *entrainer* for the *dehydration* of *ethanol* by *azeotropic distillation*. Known to be carcinogenic.

Beta-amylase (ß-amylase) An *enzyme* that *hydrolyses* the long-chain *amylose molecule*s in *starch* into fermentable *maltose*, the *dimer* (or double-*molecule*) of *glucose*. It is an *exoenzyme* in that it works from an outer end of the molecular chain. It is found in *malt* (sprouted barley) in association with α-*amylase*. With the advent of microbial *amyloglucosidase enzyme*s, malt amylases are generally only used in the production of heavily-flavored *beverage alcohol*.

Betaglucan (ß-glucan) Gum-like *polymers* of ß-linked *glucose* as in *cellulose* instead of the α-linked glucose units as in *starch* (*amylose)*. Betaglucan is commonly present in barley *mashes*. It is not broken down by α*-amylase* and causes foaming problems due to its viscous elastic nature.

Betaglucanase (ß-glucanase) An *enzyme* which *hydrolyses betaglucan*. It is frequently used in barley *mashing* to reduce foaming and viscosity problems.

Beverage alcohol Any form of distilled *ethanol* with or without *congeners* considered by law to be fit for human consumption. The laws in many countries confine beverage *alcohol* to that produced by *fermentation* as opposed to alcohol of synthetic origin.

Binary azeotrope An *azeotrope* or *constant boiling mixture* having two components such as *ethanol* and water.

Bioethanol See *fermentation ethanol.*

Biofilm A layer of living microorganisms, their polysaccharide by-products, and other soils. Biofilms form on surfaces inside process pipes and equipment and can be very potent sources of contamination.

Biogas The gas produced by *anaerobic digestion* of wastes. It is mainly *methane*.

Biological oxygen demand (BOD) A measure of the oxygen-consuming capacity of *organic* matter contained in effluents. During decomposition, organic effluents have an oxygen requirement that may deplete the supply in a waterway and result in the death of fish and other aquatic life. BOD is normally measured on the basis of the weight of oxygen required per unit volume of an effluent.

Biomass Any renewable *organic* matter such as agricultural crops, crop waste residues, wood, animal and *municipal wastes*, aquatic plants, fungal growth, etc.

Blackstrap See *molasses.*

Bleach An oxidizing agent added to detergents to enhance overall cleaning. Bleaches break down long chain protein and polysaccharide molecules. Sodium hypochlorite and hydrogen peroxide are effective bleaches used in detergent formulations.

Blended whisky Defined by the *US Bureau of Alcohol, Tobacco and Firearms* as a mixture containing at least 20% of *straight whisky* on a *proof gallon* basis, together with other *whiskies* or *neutral spirits.* When a blend contains more than 51% of a straight whisky, it may be designated by that specific type, such as 'blended rye whisky'.

Blender Tax Credit A US federal income tax credit granted to blenders of *ethanol* and *gasoline* as an alternative to the *excise tax exemption.* It was originally introduced under the *Crude Oil Windfall Profits Tax Act of 1980*, at a level of 40 cents per gallon, and was subsequently raised to 50 cents by the *Surface Transportation Assistance Act of 1982*, and to 60 cents by the *Deficit Reduction Act of 1984*.

BOD See *Biological Oxygen Demand.*

Boiler efficiency The thermal efficiency of a boiler in terms of the usable energy output (in the form of steam) in relation to the energy input in the form of fuel. Boiler efficiencies are commonly in the 70-85% range.

Boiling point The temperature at which the vapor pressure of a liquid equals the total pressure of the atmosphere above it. Bubbles of vapor can be formed when vapor pressure slightly exceeds atmospheric pressure. Boiling then takes place as heat is supplied, changing the liquid phase to the vapor phase. The boiling point of a pure substance at constant pressure does not change.

Bourbon whisky Defined by the *BATF* as *whisky* produced at not more than 160^0 *proof* from a fermented *mash* of not less than 51% corn, and stored at not more than 125^0 proof in charred, new oak *barrels*. In practice, the bourbon whisky *mash bill* is frequently about 65% corn, 25% rye, and 10% barley malt.

Brandy Defined by the *BATF* as 'an alcoholic *distillate* from the fermented juice, *mash* or wine of fruit, or from the residue thereof, produced at less than 190^0 *proof*, in such a manner that the distillate possesses the taste, aroma and characteristics generally attributed to the product'.

British Thermal Unit (BTU) The amount of heat required to raise the temperature of one pound of water one degree Fahrenheit under defined pressure conditions. It is the standard unit for measuring heat energy in the US.

Brix See *Balling.*

BTU Abbreviation for *British Thermal Unit*.

BTX Abbreviation for the three related *octane enhancers, benzene, toluene* and *xylene*.

Bubble cap A contacting device used on some *distillation plates*. It consists of a cylindrical chimney set in a hole in the plate and covered by a dome-shaped cap, which deflects the *vapors* rising up the chimney to cause them to pass through the liquid layer on the top side of the plate.

Bushel A unit of dry volume equal to 2150.42 cubic inches or 1.244 cubic feet. When used to measure grain, bushel weight depends on the type and condition of the grain. In the case of corn, bushel volume generally averages about 56 lbs by weight. This has led to the use of a *distillers bushel*, which represents 56 lbs of grain, regardless of type or volume.

Butanol (butyl alcohol) A minor constituent of *fusel oil*. Chemical formula C_4H_9OH. Four *isomers* exist. They are all colorless, toxic flammable liquids. N-Butanol may be produced as a *co-product* with acetone and *ethanol* by the *fermentation* of selected *carbohydrates* with the *anaerobic bacterium Clostridium acetobutylicum*. Butanols are used as *solvents* and chemical intermediates.

By-products Products that are secondary to the principal product of a process. In *ethanol* production, *carbon dioxide* and *distillers dried grains* are normally considered by-products; but in certain circumstances they may be viewed as *co-products*, in that they may contribute significantly to the overall process economics.

C

Carbohydrate Any of a group of compounds composed of carbon, hydrogen and oxygen including the *sugars, starches, dextrans* and *celluloses*. They are the most abundant class of *organic* compounds in nature, constituting approximately 75% of the dry weight of all vegetation.

Carbon dioxide A colorless non-flammable gas. Composition CO_2. It does not support human respiration; and in high concentrations it causes asphyxiation. It is approximately 1.5 times the weight of air, and tends to accumulate in floor drains, pits and in the bottoms of unventilated tanks. It is produced by various means, notably the combustion of fuels in an excess of air and is a *by-product* of *yeast fermentation*. It may be recovered from *fermentation*s and compressed to a liquid or solid (dry ice), or used to carbonate beverages.

Carbon monoxide A colorless, odorless, flammable gas (‘coal gas’). Composition CO. It is poisonous if inhaled, as it combines with blood hemoglobin to prevent oxygen transfer. It is very slightly lighter than air. It is produced by the incomplete combustion of fuels with a limited oxygen supply, as in auto engines. It is a major component of urban air pollution, which can be reduced by blending an oxygen-bearing compound (or *oxygenate*) such as *ethanol* into *hydrocarbon* fuels.

Carbon steel A steel deriving its particular properties from its content of carbon. These steels may range from ‘low-carbon’, (having less than 0.25% carbon), to ‘high-carbon’. Low carbon or ‘mild’ steel is easily hot-worked and rolled, and is used in steel plates and beams. It is easily welded, but is readily subject to rusting and other forms of *corrosion*.

Caribbean Basin Initiative (CBI) The program arising from the Caribbean Basin Economic Recovery Act passed by the US Congress in 1983 to encourage industrial development in the Caribbean islands and Central America. Under this program, *fuel ethanol* and other products from the region may enter the US without being subject to customs duties.

Cassava A root crop with a high *starch* content grown in the tropics and subtropical regions. Known in Brazil as *manioc*, it is used as an alternative to sugarcane as a *feedstock* for *ethanol* production. It is also processed for food as ‘tapioca’.

CBI Abbreviation for *Caribbean Basin Initiative*.

CCC Abbreviation for *Commodity Credit Corporation.*

CDA Abbreviation for *completely denatured alcohol*.

Cell recycle The process of recovering *yeast* from fermented *beer* to return it to the starting vessel of a *continuous fermentation* or to a new vessel in a *batch fermentation* system. It may include an *acid washing* step to reduce *bacterial contamination*.

Cellulase An *enzyme* capable of *hydrolyzing* long-chain *cellulose molecules* into simple *sugars* or short-chain *polymers*.

Cellulose The principal *polysaccharide* in living plants. It forms the skeletal structure of the cell wall, hence the name. It is a *polymer* of *glucose* units coupled by ß-type linkages into chains of 2,000-4,000 units. Cellulose normally occurs with other polysaccharides and *hemicelluloses* derived from sugars such as *xylose, arabinose* and *mannose*.

Celsius (or centigrade) A temperature scale in which (at normal atmospheric pressure) water freezes at zero degrees and boils at 100 degrees.

Centrifugal pump A machine for moving liquids by accelerating them radially outwards by means of a rotating impeller contained within a casing. It is the most common and most versatile type of pump in normal industrial use. It has an advantage in that it does not force a *positive displacement* of liquids, so it may be used in systems where output must be throttled or completely closed by control valves. This feature makes centrifugal pumps unsuitable for moving heavy viscous liquids such as *molasses* and syrups.

Centrifuge A machine for separating insoluble liquids or solids from liquids by the application of centrifugal force. Two main types of centrifuges are basket and decanter. In basket centrifuges, solids are retained in a rotating perforated basket while liquid passes through the perforations. In decanter centrifuges, the mixture is thrown against a solid-walled cylinder and the heavier solid particles collect against the wall for removal while the lighter liquid collects in the central part. Centrifuges are commonly used in *ethanol* plants for *yeast* recovery and in *stillage dewatering*.

Chelating agent A chemical that combines with and solubilizes water hardness ions to prevent precipitation and scale formation. Chelating agents are also used in detergent formulations to remove scale deposits. EDTA (ethylene diamine tetraacetic acid) is a chelating agent.

Chemical oxygen demand (COD) A laboratory test to determine the oxygen requirements for the chemical digestion of effluents. It should be distinguished from the *BOD* test, which determines the total of both chemical and biological oxygen requirements. COD is measured in a rapid test that involves heating the effluent in the presence of an oxidizing agent such as potassium dichromate and then determining the amount of oxygen absorbed by the effluent.

Chlorine dioxide Chemical formula ClO_2. It is a strongly-oxidizing, yellow-to-reddish-yellow gas at room temperature. It has an unpleasant odor similar to that of chlorine and reminiscent of nitric acid. It is unstable in light. It reacts violently with *organic* materials and is easily detonated by sunlight or heat in concentrations greater than 10% at atmospheric pressure. Boils at 11°C and freezes at -59°C. Chlorine dioxide may be used as a sterilant, and may be produced *in situ* for sterilizing *yeast mashes* by addition of sodium chlorite *solution* in the presence of acids or chlorine (or hypochlorite solution). It is considerably more effective as a sterilant than straight chlorine.

Chromatography A method for separating a mixture of chemical compounds into individual components by selective distribution between two immiscible materials (or phases), one stationary and the other mobile. The phases are selected so that the mobile phase will carry the various components through the stationary (or solid) phase at differing rates to give separation. *Gas chromatography* may be used to separate *ethanol* from the other *congeners* produced in fermentation and to measure them quantitatively. *High performance liquid chromatography* may be used to follow *starch hydrolysis* in *grain alcohol* production.

CIP Abbreviation for *cleaning-in-place system.*

Citrus molasses A *by-product* of the citrus juice industry. Citrus residue, mainly peel, is treated with lime and then passed through a press. The press liquor is then evaporated to a viscous, dark brown *molasses* of about 72° *Brix*. Citrus molasses is similar to cane *blackstrap molasses,* having about 45% total *sugars*. However, it has more *protein* and a much lower ash content. Citrus molasses may be diluted and fermented for *ethanol* production, but it may need pretreatment to reduce the content of d-limonene (commonly referred to as 'citrus-stripper oil'), which tends to inhibit *yeast* growth.

Cleaning In processing systems, cleaning is removing soils in the equipment to a degree that is acceptable for the process.

Cleaning-in-place system (CIP) A system designed to permit process equipment to be cleaned without disconnecting or dismantling. A sophisticated automatic CIP system may provide a sequence of water flushing cycles, detergent washing and chemical sterilizing of equipment at the press of a button.

Cleaning-out-of-place (COP) The manual disassembly, inspection and cleaning of processing equipment and systems. Some degree of COP is usually required with CIP (cleaning-in-place).

Closed receiver See *Receiver.*

CMS Abbreviation for *condensed molasses solubles.*

Coccus (plural: cocci) A type of *bacteria* with spherically-shaped cells. They may occur as single cells, clusters or long chains.

COD Abbreviation for *chemical oxygen demand.*

Column A vertical cylindrical vessel containing a series of perforated *plates* or other contact devices through which *vapors* may pass to effect a separation of liquid mixtures by *distillation.*

Co-mingled tank The term used for *fuel ethanol* tanks at refineries or pipeline terminals where two or more suppliers may share the same tank for storing *ethanol.*

Commodity Credit Corporation (CCC) An agency of the *USDA* established to stabilize and protect farm incomes and prices by maintaining balanced and adequate supplies of agricultural commodities.

Completely denatured alcohol (CDA) A term used by the *BATF* to describe *ethanol* made unfit for human consumption by addition of specified *denaturants* such as methyl isobutyl ketone, *kerosene* or *gasoline.*

Compressed yeasts A paste of viable yeasts of approximately 30% total solids prepared by filtration of yeast slurry. Refridgeration is required at 4°C, and such yeasts loose viability constantly with a shelf life of less than 3 weeks.

Concentrating column A column where *hydrous ethanol* is concentrated to decrease water content. A *beer still* may consist of a *stripping column* in which dilute *ethanol* is removed from *beer* and a concentrating column, which receives *ethanol vapor* from the stripping column and concentrates it up to about 190^0 *proof* (95^oGL). If provision is made in a concentrating column to remove impurities such as *fusel oil*, then it is more correctly a *rectifying column*.

Condensate Liquid condensed from *vapor* in a *condenser*.

Condensed molasses solubles (CMS) The term used to describe *molasses stillage* concentrated by *evaporation*. The molasses residue (after *fermentation* and *distillation*) may be concentrated to about 60° *Brix* (or approximately 60% solids) to be sold as a substitute for molasses in animal feeds as a caking agent and dust suppressant. It contains high concentrations of salts.

Condenser A *heat exchange* device connected to the *vapor* discharge pipe of a *column* to permit the vapor to be cooled and condensed to a liquid. Condensers are commonly cylindrical vessels containing tubes through which cooling water is passed.

Congeners Chemical compounds produced with *ethanol* in the *fermentation* process. They are frequently referred to as impurities. Common congeners are *methanol acetaldehyde, esters* (such as ethyl acetate) and *fusel oils* (*higher alcohols*, particularly *amyl alcohols*). *Fermentation* conditions may be adjusted to control congener formation depending on the requirements for the end product.

Constant boiling mixture See *Azeotrope*.

Continuous cooker A system into which a *mash* of water, grain and *enzyme*s may be fed continuously to be cooked and discharged to the *fermentation* system. Continuous cookers generally consist of a *slurry tank* connected by a pump to a steam jet heater (jet cooker), a holding vessel or lengths of piping (to provide some residence time at the cooking temperature), one or more flash vessels (to cool the cooked mash), a holding vessel for enzymatic *liquefaction* and a *heat exchanger* for final mash cooling. Continuous cookers are more common in *fuel ethanol* plants than in *beverage alcohol* plants.

Continuous fermentation A system into which cooked *mash* may be fed continuously to be fermented and then discharged to the *beer well* and *distillation* system. Continuous *fermentation* systems generally consist of a series of interconnected tanks sized to provide sufficient residence time for the *fermentation* to proceed to completion. The *ethanol* industry is divided on *fermentation* systems, with more gallonage produced by continuous *fermentation* than by batch *fermentation*.

Continuous distillation A process using specially-designed equipment to permit a *volatile* component such as *ethanol* to be separated by *distillation* from a continuous flow of an *aqueous solution* such as *beer*.

Control loop A portion of a process control system that includes a sensing device connected to a signal transmitter, a controller, another signal transmitter and an actuator. For example, a pressure control loop on a *distillation column* may have a pressure sensor connected through a controller to a steam flow regulating valve.

Cooker A device for heating a slurry of grain and water to a sufficiently-high temperature for sufficient time to release and *gelatinize* the *starch* in the grain and thereby render it susceptible to enzymatic *hydrolysis*. Live steam is normally used for heating the slurry; and pumps or agitators are used to ensure mixing and even heating. Cooking may be performed continuously or in a batch mode.

Cooling tower A tower or other type of structure where air (the heat receiver) circulates in direct or indirect contact with warmer water (the heat source) to cool the water. Cooling towers are used in *ethanol* production plants to recirculate cooling water and to minimize the amount of water used from wells, rivers or public sources.

Cooper A person who makes or repairs wooden *barrels*.

Cooperage A place where wooden *barrels* are made or repaired. Also used to refer to a supply of barrels (i.e., the product of the work of a *cooper*).

COP The abbreviation for *cleaning-out-of-place*.

Co-products Where the economics of a production process depend on the value of more than just the primary product, the secondary products are referred to as co-products rather than as *by-products*. For example, *distillers dried grain* may be considered a co-product of the production of *ethanol* from *dry milled* grain.

Cordials See *liqueurs*.

Corn steep liquor The concentrated liquid that has been used for steeping and softening corn prior to *wet milling*. It contains extracted low molecular weight chemicals from the corn, and serves as a nutrient source in subsequent *fermentation* of the *starch* stream.

Corn whisky Defined by the *BATF* as *'whisky* produced at under 160^0 *proof* from a fermented *mash* containing not less than 80% corn grain'. If corn whisky is stored in oak *barrels*, the *BATF* stipulates that the proof should not be more than 125^o, and that the barrels be used, or uncharred if new. Furthermore, the whisky cannot be subjected to any treatment with charred wood.

Corrosion The destruction, degradation or deterioration of material (generally metal) due to the reaction between the material and its environment. The reaction is generally chemical or electrochemical, but there are often important physical and mechanical factors in the corrosion process.

Co-solvent A liquid required to keep another liquid in solution in a third liquid. For example, in the *Dupont Waiver* for the use of 5% *methanol* in *gasoline*, 2.5% *ethanol* (or a *higher alcohol*) is required as co-solvent to improve the miscibility of the *methanol* in the gasoline.

Crude Oil Windfall Profits Tax Act of 1980 US federal legislation that extended to 1992 the e*xcise tax exemption* for *ethanol-gasoline* blends granted under the *Energy Tax Act of 1978*. It also introduced a *blender tax credit* of 40 cents per gallon of *ethanol* as an alternative to the excise tax exemption.

Cyclohexane A colorless, flammable, alicyclic *hydrocarbon* liquid of chemical formula C_6H_{12}, that boils at 80.3°C, and freezes at 6.5°C. It is used as an alternative to *benzene* as an *entrainer* in the *dehydration* of *ethanol* by *azeotropic distillation*.

D

DDG Abbreviation for *distillers dried grain*.

DDGS Abbreviation for *distillers dried grain with solubles*.

DE Abbreviation for *dextrose equivalent*.

Deadleg A length of *mash* piping closed either temporarily by a valve, or permanently to leave a dormant pocket of *mash* that may become a source of *bacterial contamination*.

Dealer tank wagon (DTW) Used in reference to *fuel ethanol* and gasoline sales, it is generally of 7,000-8,000 gallons capacity.

Decanter Vessel used for the separation of two-phase liquids. In a *fusel oil* decanter, an upper fusel oil phase is separated from a lower aqueous *ethanol* phase. In a *benzene* column *reflux* decanter, the upper, mainly-benzene, phase is separated from the lower, mainly-water, phase.

Deficit Reduction Act of 1984 US federal legislation that increased the *excise tax exemption* on *ethanol-gasoline* blends from 5 cents (as set by the *Surface Transportation Assistance Act of 1982*) to 6 cents per gallon. It also increased the alternative *blender tax* credit to 60 cents per gallon of *ethanol.*

Defoamer See *Antifoam.*

Dehydration The process of removing water from a substance, particularly the removal of most of the remaining 5% of water from 190^0 *proof ethanol* in the production of *absolute* or *anhydrous ethanol.*

Demethylizing column Occasionally referred to as a supplementary column, it is a *fractionating column* used to remove *methanol* in the production of *neutral spirit* and is located after the *rectifying column.* The demethylizing column is heated indirectly via a *reboiler.* The impure spirit enters part way up the column and the *methanol* is removed in the *overhead vapor* together with some *ethanol*, while the bulk of the *ethanol* descends to be removed at the base of the column as a product relatively free of *methanol.*

Denaturant A substance added to *ethanol* to make it unfit for human consumption so that it is not subject to taxation as *beverage alcohol.* The *BATF* permits the use of 2-5% unleaded *gasoline* (or a large numbered similar, specified substances) for use as denaturants for *fuel ethanol.* (See also *specially-denatured alcohol*).

Department of Energy (DOE) A department of the US federal government established in 1977 to consolidate energy-orientated programs and agencies. The department's mission includes the coordination and management of energy conservation, supply, information dissemination, regulation, research, development and demonstration. The department includes an *Office of Alcohol Fuels.*

Dephlegmator Name commonly used for the first of two or more *condensers* attached to the *overhead vapor* line of a *distillation column.* It literally means an entrained liquid separator.

Desiccant A substance that absorbs water and can be used for drying purposes. For example, *potassium aluminosilicate* is used as a desiccant in *molecular sieve* systems for *ethanol dehydration.*

Detergent package A combination of detergents and *corrosion inhibitors* normally added to *fuel ethanol* to impart a cleaning action to *ethanol-gasoline* blends and thereby reduce engine fuel injector blockages.

Dewatering The removal of water (or other liquids) from a solid material, particularly the use of screens or centrifuges in the initial separation of *thin stillage* (liquid) from the solids contained in *whole stillage* in *DDG* processing.

Dextran A non-fermentable, large, branched-chain *polymer* of glucose *molecule*s produced from sucrose in *molasses* by *bacterial contamination* (mainly *Leuconostoc mesenteroides*). It gives a ropey appearance to molasses when stirred or poured, and reduces *ethanol* yield on *fermentation.*

Dextrin Short-chain *polymers* of *glucose molecule*s produced by the partial *hydrolysis* of *starch* with *α-amylase* or acid in the initial stage of conversion to *fermentable sugars.*

Dextrose An alternative name for *glucose.*

Dextrose equivalent (DE) A measure of the degree of *hydrolysis* of *starch.* It is no longer considered as significant as previously in *ethanol* production with the more general acceptance of *simultaneous saccharification and fermentation.* The use of HPLC to measure fermentable carbohydrate directly makes DE determination necessary.

Diethyl ether The traditional anaesthetic *ether*, which is employed as an *entrainer* in some *azeotropic distillation* processes for *fuel ethanol dehydration* (as an alternative to the more commonly-used *benzene*). It is a colorless, flammable liquid that boils at 34.6°C and freezes at -117°C. Chemical formula $(C_2H_5)_2O$. It readily forms explosive mixtures with air.

Differential pressure cell (DP cell) A device for measuring the difference in pressure of liquid on either side of a restricting orifice. It is used in flow measurement. (See *Orifice meter)*.

Dimer A compound produced by linking together two *molecule*s of a simpler compound (or *monomer*). It is the simplest form of *polymer*. For example, *maltose* is a dimer composed of two linked *glucose* molecules.

Disaccharide A compound *sugar* that yields two *monosaccharide* units on *hydrolysis*. For example, *lactose* yields *glucose* and *galactose, sucrose* yields *glucose* and *fructose*, while *maltose* yields two *glucose* units.

Disc and donut column A *beer distillation tower* patented by Raphael Katzen with *trays* alternately consisting of annular shelves with open centers (donuts) and discs (of a slightly larger diameter than the donut holes) placed centrally below the holes. The effect is to give a circular curtain of liquid descending from tray to tray, through which the rising *vapors* must pass. It has the advantage of being unaffected by the solids content of the *beer* feed or any *scaling* that may occur in a typical tray column.

Distilland Material to be distilled, such as *beer*.

Distillate The portion of a liquid (*distilland)* removed as a *vapor* and condensed during a *distillation* process.

Distillation The process by which the components of a liquid mixture are separated by differences in *boiling point* by boiling and recondensing the resultant *vapors*. In *ethanol* production, it is the primary means of separating *ethanol* from *aqueous solutions*.

Distilled spirits permit (DSP) A permit issued by the *BATF* allowing the holder to engage in the production or warehousing of undenatured *ethanol* for beverage, industrial or fuel use.

Distillers bushel 56 lbs of any grain, regardless of volume.

Distillers dried grain (DDG) The dried residual *by-product* of a grain *fermentation* process. It is high in protein, as most of the grain *starch* has been removed. It is used as an animal feed ingredient. By strict definition, DDG is produced only from the solids separated from *whole stillage* by *centrifuging* or screening. In practice, the term is commonly used to describe the entire dried stillage residue, making it synonymous with *DDGS.*

Distillers dried grain with solubles (DDGS) The product derived by separating the liquid portion (*thin stillage* or solubles) from grain *whole stillage* by screening or *centrifuging*, then *evaporating* it to a thick syrup and drying it together with the grain solids portion.

Distillers dried solubles The product derived from separating the liquid portion (*thin stillage*) from grain *whole stillage*, *evaporating* it to a thick syrup and then drying it to a fine powder.

Distillers feeds The *by-products* of *fermentation* of cereal grains. See *DDG* and *DDGS*.

Distillers wet grain (DWG) The wet grain residue separated from *whole stillage* by screening or *centrifuging*. It has a very limited storage life and is generally used only where cattle feedlots are located near *ethanol* production plants.

Distillery A building or premises where alcohol is distilled.

Distillery run barrels Recently-emptied, used *whiskey barrels* that have not been sorted to remove those with or without defects.

DMA 67Y A detergent produced by Dupont. It is added to *fuel ethanol* to ensure that the resultant blend with *gasoline* will have clean-burning characteristics similar to detergent *gasoline*s supplied by major oil companies.

Downcomer (or downpipe) A device used in *distillation columns* to allow liquid to descend from one *plate* to another. Downcomers may take the form of one or more round, oval or rectangular section pipes, or chordal baffles. The downcomer usually has a weir-type seal at the bottom to prevent *vapors* passing upward.

DP cell See *differential pressure cell.*

Dry degermination A process for the removal of germ from grain without the need for steeping and *wet milling*. It may involve some pretreatment of the grain to raise the moisture content before processing. It is used in Scotland for corn milling in *grain whisky* plants and is used in the production of corn flakes; but is not commonly used in the US *ethanol* production industry.

Dry milling In the *ethanol* production industry dry milling refers to the milling of whole dry grain where, in contrast to wet milling, no attempt is made to remove fractions such as germ and bran. Milling may be carried out with various types of equipment, including *hammer mills* and *roller mills.*

DSP Abbreviation for *Distilled Spirits Permit.*

DTW Abbreviation for *Dealer Tank Wagon.*

Dual-flow plate (or tray) A perforated *plate* similar to a *sieve plate*, without *downcomers.* The perforations are of such a size and open area that the liquid descends by *weeping* through the holes.

Dunder A Caribbean synonym for *vinasse* or *molasses stillage*. It is commonly used to refer to vinasse that has been stored for some time to allow bacterial development prior to being used as *backset* in the production of heavily-flavored *rums*.

Dupont waiver The waiver received by Dupont from the *EPA* in 1985 for a blend of *gasoline* containing a maximum of 5% *methanol* plus a minimum of 2.5% *ethanol* or other approved *co-solvent*, together with an approved proprietary *corrosion inhibitor.*

DWG Abbreviation for *distillers wet grain.*

E

Ebulliometer A laboratory instrument used to measure alcohol concentration in water-ethanol mixtures. The mixture is heated to boiling and the temperature of the vapor is measured accurately and corrected for barometric pressure. The ethanol concentration is read from a table of concentration *vs* boiling point.

ED Abbreviation for *extractive distillation.*

Effect In the context of *evaporators*, the term is used to describe one vessel in a series. For example a quadruple effect *evaporator* consists of four linked vessels.

EITC Abbreviation for *energy investment tax credit.*

Endoenzyme An *enzyme* acting on internal portions of a large *polymeric molecule* rather than around the periphery. For example, the *α-amylase enzyme hydrolyzes* linkages within *amylose* and *amylopectin molecule*s. In contrast, *amyloglucosidase* acts as an *exoenzyme* by only hydrolyzing the outermost linkages.

Energy Policy Act of 1992 US federal legislation that amended the provisions of the Omnibus Reconciliation Act of 1990 to allow the proration of the *excise tax exemption* of 5.4 cents per *gallon* on *ethanol gasoline* blends that use less than the standard content of 10% *ethanol*. This was to meet the requirements of the Clean Air Amendments Act that required that oxygenate blends with 2.1% or 2.7% oxygen be used in *gasoline*s in designated areas suffering from severe air pollution. (These oxygen rates correspond to *ethanol* usages of 6% and 7.7%, respectively).

Energy Security Act of 1980 US federal legislation that supported the emerging *fuel ethanol* industry by establishing an independent *Office of Alcohol Fuels* within the *Department of Energy* and authorized programs of *loan guarantees*, price guarantees and purchase agreements with fuel *ethanol* producers.

Energy Tax Act of 1978 US federal legislation that instituted the first *excise tax exemption* for *gasoline* blended with 10% *fermentation ethanol*. It exempted the blends from the tax of 4 cents per gallon. It also created an *energy investment tax credit (EITC)* of 10% that applied to equipment for converting *biomass* to *ethanol* in addition to the standard 10% investment tax credit.

Entrainer A substance used to assist in the *dehydration* of *ethanol* by *azeotropic distillation*. Examples are *benzene, cyclohexane* and *pentane*. (See *azeotropic distillation*).

Environmental Protection Agency (EPA) A US government agency established in 1970. EPA responsibilities include the regulation of fuels and fuel additives, including *ethanol gasoline* blends.

Enzymatic hydrolysis The *hydrolysis* of a *polymer* by the use of *enzyme*s. In the case of *starch* hydrolysis, an α-*amylase enzyme* may be used in the initial hydrolysis to achieve *liquefaction* and an *amyloglucosidase enzyme* may be used to complete the hydrolytic *saccharification* to *fermentable sugars*.

Enzyme Any of a class of complex proteinaceous substances (such as amylases and lactases) produced by living organisms that catalyze chemical reactions without being destroyed. Enzymes may act outside the producing organism and maybe used in industrial processes such as saccharification.

EPA Abbreviation for *Environmental Protection Agency*.

Ester The product derived by the reaction of an acid with an *alcohol* or other *organic* compound having *hydroxyl* groups. For example, *ethyl acetate* is an ester produced by reacting *acetic acid* with *ethanol*. Esters tend to accumulate in *distillation* in the *heads* at the top of the tower.

ETBE Abbreviation for *ethyl tertiary butyl ether*.

Ethanol Otherwise known as *ethyl alcohol, alcohol*, grain-spirit or *neutral spirit*, etc. A clear, colorless, flammable *oxygenated hydrocarbon*. Chemical formula: C_2H_5OH. It has a *boiling point* of 78.5°C in the *anhydrous* state. However, it forms a *binary azeotrope* with water with a boiling point of 78.15°C at a composition of 95.57% by weight *ethanol*.

Ether One of a class of *organic* compounds in which an oxygen atom is interposed between two carbon atoms in the *molecular* structure. Ethers may be derived from *alcohols* by the elimination of water. Ethers such as *diethyl ether* and *isopropyl ether* may be used as *entrainers* in the *dehydration* of *ethanol* by *azeotropic distillation*. Other ethers such as *MTBE* and *ETBE* may be used as *octane enhancers* in *gasoline*. Ethers are dangerous fire and explosion hazards. When exposed to air they form peroxides that may detonate on heating.

Ethyl acetate Chemical formula $CH_3COOC_2H_5$. A clear, volatile, flammable liquid with a characteristic fruity odor. It has a pleasant, sweet taste when diluted. Boils at 77°C and freezes at -83°C. It is produced by the reaction of *ethanol* with *acetic acid* and is a major component of the *esters* that give *rum* its characteristic odor.

Ethyl alcohol See *Ethanol*.

Ethyl carbamate Otherwise known as *urethane* or carbamic acid ethyl ether. A carcinogenic compound produced by heating urea with *ethanol* under pressure. Chemical formula $NH_2COOC_2H_5$. It is very soluble in *ethanol* or water. Traces of ethyl carbamate may be formed in the *distillation* of *beers* produced with the use of urea as a *yeast* nutrient. It does not present a significant hazard if such *ethanol* is only used for fuel purposes, but may present problems in the production of *whiskies*.

Ethylene glycol A slightly viscous, sweet-tasting, poisonous liquid. Chemical formula CH_2OHCH_2OH. It has a *boiling point* of 197.6°C, and is considerably *hygroscopic*. It is commonly used as an antifreeze. It may also be used as an *extractant* in *extractive distillation* processes for the *dehydration* of *ethanol*. (See *extractive distillation*).

Ethyl tertiary butyl ether (ETBE) A colorless, flammable, *oxygenated hydrocarbon*. Chemical formula $C_2H_5OC_4H_9$. It may be produced from *ethanol* and tertiary *butanol (TBA)* or isobutylene. It is of similar structure to *methyl tertiary butyl ether (MTBE)*, having similar *octane-enhancing* properties, but has a significantly lower effective *Reid vapor pressure* in blending with *gasoline*.

Evaporation The process by which a substance in the liquid state is converted into the *vapor* state.

Evaporator A device used to evaporate part or all of a liquid from a *solution*. In the case of grain *stillage* processing, evaporators may be used to concentrate screened *thin stillage* to a syrup of about 35% solids, which may then be fed to a dryer. Evaporators are normally operated under vacuum (to obtain a lower *boiling point* of the liquid) and may consist of a series of interlinked vessels or *effects* operating under differing degrees of vacuum.

Extractant A substance such as *ethylene glycol* or *glycerol* be used in *extractive distillation* processes for the *dehydration* of *ethanol*.

Extractive distillation A process where an *extractant* is added to a mixture being distilled to change the *volatility* of one or more components. The less *volatile* mixture will then descend in a *continuous distillation column* while the more volatile components may be removed in the condensed *overhead vapors*. In the use of extractive distillation in the *dehydration* of *ethanol*, liquid extractants such as *ethylene glycol,* or *glycerol,* may be used. Salts such as potassium and sodium acetates may also be used alone in molten form or in mixtures with glycerol, etc. *Anhydrous ethanol* is recovered in the overhead *condensate* while the water combines with the extractant to emerge from the bottom of the column. (This is the reverse of the situation in *azeotropic distillation*). The extractant is then separated from the water in another column (or an evaporator) and is recycled.

In the production of *neutral spirit*, light *rums* or *whiskies*, extractive distillation may be used to remove *fusel oils* and some other *congeners* in the condensed *overhead vapors*. In this instance the extractant is water, as some of the congeners with lower *volatility* than *ethanol* in a concentrated state may have higher volatility than *ethanol* when diluted with water and therefore rise up the extractive distillation column while the *ethanol* descends.

Exoenzyme An *enzyme* restricted to acting on the outer end of large *polymer*ic *molecule*s and cleaves molecules one by one. (See *Endoenzyme*).

F

Facultative anaerobe Term used to describe a microorganism (such as a *yeast*) that is essentially aerobic (or oxygen-requiring), but can also thrive under *anaerobic* (or oxygen-free) conditions.

Fahrenheit scale A temperature scale in which the *boiling point* of water is 212°F and the freezing point is 32°F. (The zero point was originally established as the lowest point obtainable with a mixture of equal weights of snow and common salt).

Feed plate (or feed tray) The *plate* or *tray* onto which the *distilland* (liquid to be distilled) is introduced in a *distillation tower*. In theory, it is the point in a tower above which enrichment or concentration occurs and below which stripping occurs.

Feedstock The raw material used in a process. For example, corn, *molasses, whey,* etc. may be used as feedstocks for *ethanol* production.

Fermentable sugars Simple *sugars* such as *glucose* and *fructose* that can be converted into *ethanol* by *fermentation* with *yeast*. They may be derived by the *hydrolysis* of *starch* or *cellulose feedstocks* or obtained from other sources.

Fermentation The enzymatic transformation by *microorganisms* of *organic* compounds such as *sugars*. It is usually accompanied by the evolution of gas as in the *fermentation* of *glucose* into *ethanol* and *carbon dioxide*.

Fermentation efficiency The measure of the actual output of a *fermentation* product such as *ethanol* in relation to the *theoretical yield*.

Fermentation ethanol The term used to distinguish *ethanol* produced by *fermentation* from *synthetic ethanol* produced from ethylene, etc. The difference is significant in that only *fermentation ethanol* qualifies for US federal and state *excise tax exemptions* for automotive fuel use. Furthermore, only *fermentation ethanol* may be used for beverage purposes, and in many countries to make vinegar.

Fermentor The vessel in which *mash fermentation* takes place. The vessel may be fabricated from stainless steel, fiber glass, etc. and is normally fitted with an internal or external cooling system for controlling temperature of the fermenting mash.

FFV Abbreviation for *Flexible Fueled Vehicle*.

Flame arrester A device installed on the vapor vents of *alcohol* storage tanks and *distillation columns* to prevent entry of flames that might cause an explosion. It normally contains fine metal meshes with holes large enough to allow vapors to escape, but too small to allow flames to pass in the opposite direction.

Flash cooling The rapid cooling achieved when a hot liquid is subjected to a sudden pressure drop to reduce its *boiling point*.

Flash point The minimum temperature at which a combustible liquid will ignite when a flame is introduced. It depends on the *volatility* of the liquid to provide sufficient *vapor* for combustion. For example, *anhydrous ethanol* has a flash point of 51°F, while 90^0 *proof* ethanol has a flash point of 78°F.

Flexible fueled vehicle (FFV) A vehicle designed to operate on a variety of fuels such as *methanol*, *ethanol* or *gasoline* alone or in combinations without requiring major adjustments.

Flocculation The aggregation or coalescing of fine suspended particles or bodies into loose clusters or lumps. The flocculation characteristics of *yeast* or other *microorganisms* may be important features in their recovery for *cell recycling*.

Flow meter A device for measuring the rate of flow of a fluid.

Fluidized bed combustion boiler A boiler in which the coal or other fuel particles are kept in suspension by a rising column of gas rather than resting on a conventional grate. The system gives greater heat transfer and higher potential combustion efficiency. The system has the advantage that limestone may be added to the coal fuel to absorb sulfur gases to reduce emissions.

Fossil fuel Any naturally-occurring fuel of an *organic* nature that originated in a past geologic age such as coal, crude oil or natural gas.

Fractional distillation A process of separating mixtures such as *ethanol* and water by boiling and drawing off the condensed *vapors* from different levels of the *distillation tower*.

Fructose A fermentable *monosaccharide* (simple *sugar*) of the chemical formula $C_6H_{12}O_6$. Its chemical structure is similar to that of *glucose*, but it is sweeter to the taste. It may be produced from *glucose* by enzymatic *isomerization*, as in the production of *high fructose corn syrup (HFCS)*.

Fuel grade ethanol See *motor fuel grade ethanol*.

Fuel ethanol Usually denotes *anhydrous ethanol* that has been *denatured* by addition of 2-5% unleaded *gasoline* and is intended for use as an automotive fuel in blends with *gasoline*.

Fungible Literally means ‘interchangeable in trade’. Commonly used to denote products suitable for transmission by pipeline. *Ethanol* is not considered fungible in this sense because it would absorb any water accumulating in pockets in a pipeline.

Fusel oil Term used to describe the *higher alcohols*, generally the various forms of *propanol, butanol* and *amyl alcohol* that are *congeners* or *by-products* of *ethanol fermentation*. Normally, predominantly isoamyl alcohol. Their presence in alcoholic beverages is known to be a cause of headaches and hangovers. The fusel oils have higher *boiling point*s than *ethanol* and are generally removed in the *distillation* process to avoid accumulation in the *rectifier.* They may be subsequently added back into the *anhydrous* product for *motor fuel grade ethanol*.

Fusel oil decanter A device used to separate accumulations of *fusel oil* from *ethanol* based on the fact that fusel oil has a lower miscibility in water than *ethanol* and can therefore be removed by dilution with water. It generally consists of a tank with windows or sight glasses to permit the operator to observe and control the separation.

G

Galactose A *monosaccharide* of chemical formula $C_6H_{12}O_6$ that along with *glucose* is a constituent of the *disaccharide lactose*. It is an *isomer* of *glucose*, but is less-readily fermented by *yeasts* to *ethanol*.

Gas chromatography (GC) A technique for separating chemical substances in which the sample is carried by an inert gas stream through a tube (or column) packed with a finely-divided solid material. The various components in the sample pass through the column at differing velocities and emerge from the column at distinct intervals to be measured by devices such as a flame ionization detector or a thermal conductivity detector. (Where the solid material in the column is pretreated with a liquid to achieve the component separation, the process may be referred to as *gas liquid chromatography*). The technique may be used for separating and measuring the amount of *ethanol* and the various *by-products* formed in *fermentation*.

Gas liquid chromatography See *gas chromatography.*

Gasohol (or gasahol) A trade name registered by the Nebraska Agricultural Products Industrial Utilization Committee, later renamed the *Nebraska Gasohol Committee*. (The committee was responsible for laying the groundwork for the development of the present day US *fuel ethanol* industry). The trade name, in either spelling, covers a blend of *anhydrous* ethanol 'derived from agricultural products' with *gasoline* (not necessarily unleaded). The committee has freely granted permission for commercial use of the trade name provided it is not used for blends containing *alcohols* other than *ethanol*.

Gasoline A volatile, flammable, liquid *hydrocarbon* mixture suitable for use as a fuel in internal combustion engines. Normally consists of a blend of several products from natural gas and *petroleum* refining together with anti-knock agents and other additives. It is a complex mixture of hundreds of different hydrocarbons, generally in the range of 4-12 carbon atoms per *molecule*. The components may have *boiling points* ranging mainly between 30 and 200°C with blends being adjusted to altitude, season and legal requirements.

Gasoline extender The term used to describe *ethanol* when it is simply used as a partial replacement for *gasoline* without any consideration for its value as an *octane enhancer* or oxygenate.

Gay Lussac (GL) The name given to a scale of the concentration of *ethanol* in mixtures with water where each degree is equal to 1% by volume (i.e., 1° GL is equivalent to 2° US *proof*). It takes the name from the French chemistry pioneer, Joseph-Louis Gay Lussac.

Gay Lussac equation The equation for the *fermentation* of *sugar* by *yeast* to *carbon dioxide* and *ethanol*, established by the French chemist Joseph-Louis Gay Lussac in 1815: $C_6H_{12}O_6 \rightarrow 2CO_2 + 2C_2H_5OH$. (See *Stoichiometric yield)*.

GC Abbreviation for *gas chromatograph*.

Gear pump A *positive displacement* rotary pump containing two intermeshed gear wheels in a suitable casing. The counter rotation of the gears draws fluid between the gear teeth on one side and discharges it on the other side. It is commonly used for viscous liquids such as *molasses* and *stillage* syrup.

Gelatinization In reference to the cooking of starchy *feedstocks*, gelatinization is the stage in which the *starch* granules absorb water and lose their individual crystalline structure to become a viscous liquid gel. Gelatinization is significant in that it is the preliminary process necessary to render *starch* susceptible to *enzymatic hydrolysis* for conversion to *fermentable sugars*.

Gin Defined by the *BATF* as 'a product obtained by original *distillation* from *mash*, or by redistillation of distilled spirits, or by mixing *neutral spirits* with or over juniper berries and other aromatics, or with or over other extracts derived from infusions, percolations or maceration of such materials, and includes mixtures of gin and neutral spirits. It shall derive its main characteristic flavor from juniper berries and be bottled at not less than 80^0 *proof*. Gin produced exclusively by original distillation or redistillation may be further designated as distilled'.

GL Abbreviation for *Gay Lussac*.

GLC Abbreviation for *gas-liquid chromatography*.

Glucoamylase An *enzyme* that *hydrolyses starch* into its constituent *glucose* units. (See *Amyloglucosidase*).

Glucan See *Betaglucan*.

Glucanase An *enzyme* that *hydrolyses* glucan. (See *Betaglucanase*).

Glucose A *fermentable sugar* otherwise referred to as *dextrose*. It is a *monosaccharide* and has the formula $C_6H_{12}O_6$. Glucose is the ultimate product in the *hydrolysis* of *starch* and *cellulose*, which are both *polymers* of hundreds or thousands of *glucose* units.

Glucose isomerase An *enzyme* that converts *glucose* into its *isomer, fructose*. It is used in the production of *high fructose corn syrup (HFCS)*.

Glucosidase See *Amyloglucosidase* or *Glucoamylase*.

Glycerol (or glycerine) A clear, colorless, viscous, sweet-tasting liquid belonging to the *alcohol* family of *organic* compounds. It has a chemical formula of $CH_2OHCHOHCH_2OH$, having three *hydroxyl* (OH) groups. It is a *by-product* of alcoholic *fermentation*s of *sugars*. It is *hygroscopic* and may be used as an *extractant* in the *dehydration* of *ethanol*.

Grain alcohol Term used to distinguish *ethanol* produced from grain from *ethanol* produced from other *feedstocks*, or from *wood alcohol* (*methanol)*.

Grain whisky The term used in Scotland to distinguish the bland, nearly-neutral, continuous *distillation* product that forms the base of blended Scotch whisky from the *malt whiskies* that contribute most of the flavor in the blend. Grain whisky is usually produced from corn or barley.

Grain sorghum Otherwise known as *milo*, a sorghum grown for grain production, as distinct from *sweet sorghum* grown for the *sugar* content of its stem. It may be used as a *feedstock* for *ethanol* production.

Gram-negative See *Gram stain*.

Gram-positive See *Gram stain*.

Gram stain A widely-used microbiological staining technique that aids in the identification and characterization of *bacteria*. It was devised by Danish physician, Hans Christian Gram. Bacteria are described as *Gram-positive* if their cell walls absorb and retain the stain and *Gram-negative* if they do not. The technique is particularly useful in examining *bacterial contaminants* in *fermentations*, as most Gram-positives are susceptible to control by *penicillin*.

H

Hammer mill A type of impact mill or crusher in which materials such as cereal grains are reduced in size by hammers revolving rapidly in a vertical plane within a steel casing. A screen with numerous holes of a selected diameter is installed in the casing to control the size of particles produced. It is commonly used for grinding corn as a *fermentation* feedstock.

Heads Term used to describe the impurities produced in *ethanol fermentations* (*congeners*) that have lower *boiling point*s than *ethanol*. They include *methanol* and *aldehydes*.

Heads concentrating column A *distillation column* used to concentrate *heads* removed in the production of *neutral spirit*, light *rums* and *whiskies*.

Heat exchanger Device used to transfer heat from a fluid on one side of a barrier to another fluid flowing on the other side of the barrier. Common forms include shell-and-tube heat exchangers and plate-type heat exchangers. Various types of heat exchangers are used in *ethanol* plants for *mash* cooling, fermentation temperature control, indirect steam heating (*reboilers*), *overhead vapor* condensing, etc.

Heat of condensation The heat given up when a *vapor* condenses to a liquid at its *boiling point*.

Heat of vaporization The heat input required to change a liquid at its *boiling point* to a *vapor* at the same temperature.

Hemicellulose Term used to describe non-cellulosic *polysaccharide* components of plant cell walls. The most common hemicelluloses are composed of *polymers* of *xylose* (a 5-carbon sugar, or *pentose*) together with a uronic acid (a sugar acid), *arabinose* (another pentose) and *mannose* (a *hexose*). Hemicelluloses have no chemical relationship to *cellulose*. They frequently surround the cellulose fibers and increase their bonding and tensile strength. The presence of hemicelluloses (and *lignins*) in close association with cellulose tends to impede the extraction and *hydrolysis* of wood cellulose to *sugars* for *ethanol* production, as well as its degradation in nature.

Hexose A class of *monosaccharides* (simple *sugars*) containing six carbon atoms in the *molecule*. They have the formula $C_6H_{12}O_6$. Common examples are *glucose*, *fructose* and *galactose*.

HFCS Abbreviation for *high fructose corn syrup*.

Hiag process A process developed in Germany in the 1930s for the *dehydration* of *ethanol* by *extractive distillation* using a mixture of sodium and potassium acetates as the *extractant*.

High boilers Term used to describe the impurities produced in *ethanol fermentation* (*congeners*) that have higher *boiling point*s than *ethanol*. Otherwise referred to as *tails*, they include the *higher alcohols*, *propanols, butanols* and *amyl alcohols* or *fusel oils*. In the context of *gasoline*, the term refers to components with *boiling point*s considerably above the mid-range.

Higher alcohols *Alcohols* having more than two carbon atoms. They exist in various isomeric forms. As the number of carbon atoms increases, so does the number of *isomers*, but at a greater rate. The lower members of this group, namely *propanol, butanol* and *amyl alcohol* are major constituents of *fusel oil*.

High fructose corn syrup (HFCS) A product in which a large percentage of the *glucose* derived from *starch hydrolysis* has been converted into its sweeter-tasting *isomer fructose* by use of *enzyme*s. It is frequently used as a substitute for cane or beet *sugar* as a sweetener in soft drinks, etc. It is a seasonal alternative to *ethanol* production in some corn processing plants.

High performance liquid chromatography (HPLC) An advanced form of liquid *chromatography* used for the separation of complex mixtures of non-volatile materials for analytical purposes. It involves very small particles packed in columns through which the liquids flow and high pressures to increase the rate of flow and shorten the time required to achieve the separation. The process is coupled with other devices to identify the constituents. It may be used in the analysis of the *sugars* and *dextrins* produced by *hydrolysis* of *starch*.

High test molasses (HTM) See *molasses*.

Hogshead A wooden *barrel* with a capacity of approximately 66 *US gallons* (250 *liters*) used in Scotland for aging *whisky*. It is usually constructed from *shooked bourbon whiskey* barrels of 52 gallons capacity by using additional staves and larger heads and hoops.

HPLC Abbreviation for *high performance liquid chromatography*.

HTM Abbreviation for *high test molasses*.

Hydrocarbon A member of a class of *organic* chemical compounds containing only hydrogen and carbon. It should be clearly differentiated from *carbohydrates*, which contain oxygen as well as hydrogen and carbon. The main natural sources of hydrocarbons are *petroleum*, coal, natural gas and bitumens.

Hydrolysis Literally means the breakdown, destruction or alteration of a chemical substance by water. In the case of *starch* and other *polymers* of *glucose*, a *molecule* of water is divided between two adjacent *glucose* units, in order to cleave the linkage. For example, *maltose* ($C_{12}H_{22}O_{11}$), which contains two *glucose* rings, requires the addition of a *molecule* of water (H_2O) to yield two separate *glucose molecule*s ($C_6H_{12}O_6$). The hydrolysis may be accomplished with the use of acids or *enzyme*s.

Hydrometer An instrument measuring the density, *specific gravity* or other similar characteristics of liquids. It is generally comprised of a long-stemmed glass tube with a weighted bottom which floats at different levels in liquids of different densities. The reading is taken at the meniscus (where the calibrated stem emerges from the liquid). The liquid temperature is normally determined when taking a reading; and reference is made to hydrometer tables to obtain a correction to a standard temperature. A *proof* hydrometer measures the content of *ethanol* in a mixture with water. A *Brix* or *Balling* hydrometer measures on a scale equivalent to the percentage of *sugar* by weight in an *aqueous solution.*

Hydroselection column Synonym for *extractive distillation column.*

Hydrous ethanol Term used for *ethanol* that has not been subjected to *dehydration.* It may refer to any mixture of *ethanol* and water, but frequently is used to denote *ethanol* at a concentration of about 190-192^0 *proof*, close to the *azeotropic* point.

Hygroscopic Term used to describe a substance with the property of absorbing moisture from the air. *Anhydrous ethanol* is hygroscopic, and its exposure to moist air should therefore be minimized.

Hydroxyl group A combination of one atom of oxygen and one atom of hydrogen (OH) forming an essential part of any *alcohol.* The spare atomic bond of the group is linked to a carbon atom as in *ethanol* (C_2H_5OH) or *methanol* (CH_3OH).

I

IDRB Abbreviation for *Industrial Development Revenue Bonds.*

Imperial gallon A measure of volume in the British system defined in 1824 as the volume occupied by 10 pounds of water at 62°F and 30 inches of barometric pressure. It is the equivalent of 1.2 US gallons, or 4.546 liters.

Industrial alcohol Denotes any *ethanol* intended for industrial uses such as *solvents, extractants*, antifreezes and intermediates in the synthesis of innumerable organic chemicals. The term covers *ethanol* of both *synthetic* and *fermentation* origin of a wide range of qualities and *proofs*, with or without *denaturants.*

Industrial development revenue bonds (IDRB) Debt incurred through local industrial development authorities in numerous US states. Such bonds were a popular way to finance *fuel ethanol* plants in the past because the interest on the bonds was not subject to income tax and the bonds could be sold with interest rates substantially below the prime rate.

Inoculum The portion of a culture of *yeast* (or *bacteria*) used to start a new culture or a *fermentation.*

Inulin A storage *polysaccharide* found in the roots and tubers of various plants, particularly Jerusalem artichokes. It consists of chains of an average of 30 *fructose* units. It is only slightly soluble in cold water, but dissolves readily in hot water. Unlike *starch*, it does not give a color reaction with iodine.

Inulinase An *enzyme* capable of *hydrolyzing inulin* to its component *fructose* units.

IPE Abbreviation for *isopropyl ether.*

Isoamyl alcohol The principal *alcohol* in *fusel oil*. It is an *isomer* of *pentanol*, of composition $C_5H_{11}OH$. It is a colorless liquid with a pungent taste and disagreeable odor. Boils at 132°C and freezes at -117.2°C. It is only slightly soluble in water but miscible with *ethanol.* It may be recovered by fractionation of *fusel oil*, and has a wide range of uses in *organic* synthesis, pharmaceuticals, photographic chemicals and as a *solvent* for fats, etc. The *vapors* are poisonous and at low concentrations may cause headaches and dizziness.

Isomer One of a series of two or more *molecule*s with the same number and kind of atoms and hence the same *molecular weight*, but differing in respect to the arrangement or configuration of the atoms. For instance, *glucose* and *fructose* have the formula $C_6H_{12}O_6$, but different molecular structures.

Isomerase An *enzyme* that can convert a compound into an isomeric form. For instance, the *glucose isomerase enzyme* converts *glucose* into its sweeter-tasting *isomer fructose* in the production of *high fructose corn syrup (HFCS).*

Isomerization The process of converting a chemical compound into its *isomer* such as converting *glucose* to *fructose* in the production of *high fructose corn syrup (HFCS*). In *petroleum* refining, the term describes methods used to convert straight-chain to branched-chain *hydrocarbons*, or acyclic to aromatic hydrocarbons to increase their suitability for high *octane gasoline*.

Isopropyl ether (IPE) An ether (otherwise known as di-isopropyl ether) used in some *fuel ethanol* plants as an *entrainer* in the *dehydration* process as an alternative to *benzene,* etc. It is a colorless, volatile liquid with chemical formula $(CH_3)2CHOCH(CH_3)_2$, which boils at 67.5°C and freezes at -60°C. It readily forms explosive mixtures with air. Inhalation of vapors may cause narcosis and unconsciousness.

J

Jet cooker An apparatus for the *continuous cooking* of grain *mashes* in which the mash is pumped past a jet of steam that instantly heats the mash to *gelatinize* the *starch*.

Jobber A *gasoline* wholesaler. Some jobbers may operate under contract to one or more major oil companies, distributing *gasoline* to branded gas stations. Other jobbers may operate independently, buying *gasoline* from various sources for distribution to unbranded retail outlets. Independent jobbers are frequently major buyers and blenders of *fuel ethanol.*

K

Karl Fischer titration A method to chemically determine the amount of water present in a sample of *ethanol* and/or other substances. When correctly practiced, the method provides an extremely accurate measurement of very small quantities of water in *ethanol* even if *gasoline denaturant* is present. (See *titration).*

Kerosene One of the three permissible *denaturants* for *fuel ethanol* as specified in *BATF* regulations. It is a refined *petroleum* fraction used as a fuel for heating, cooking and for jet engines. It has a *boiling point* range somewhat higher than that of *gasoline*, generally between 180°C and 290°C.

Kjeldahl method An analytical method for the determination of nitrogen in *organic* compounds. As nitrogen is an essential element in *protein*, the method may be used for determining protein in such materials as *distillers dried grains*. Kjeldahl protein, or crude protein, is Kjeldahl N x 6.25.

Kluyveromyces fragilis (or marxianus) A *lactose*-fermenting *yeast* used in the production of *ethanol* from cheese *whey*.

Kubierschky process The first patented process for the continuous *dehydration* of *ethanol* with *benzene*. With relatively minor variations, the process developed in 1914 based on Young's earlier batch process is still used today in *fuel ethanol* plants.

L

Lactase An *enzyme* that *hydrolyzes lactose* into *glucose* and *galactose*. This hydrolysis allows lactose-containing *feedstocks* such as cheese *whey* to be fermented by the common *Saccharomyces cerevisiae yeasts*. A principal source of lactase is the yeast *Kluyveromyces fragilis*, which can also directly ferment lactose to *ethanol*.

Lactic acid The *organic* acid produced in the *fermentation* of carbohydrates by *Lactobacillus bacteria*. Its production is the principal reason for loss of yield in contaminated *ethanol fermentation*s. Pure lactic acid is a colorless, odorless, *hygroscopic*, syrupy liquid with a *boiling point* of 122°C and a formula $CH_3CHOHCOOH$.

Lactobacillus A genus of *bacteria* that produce *lactic acid* as a major product in the *fermentation* of *carbohydrates*. Lactobacilli are found extensively in fermenting food products such as souring milk and in grain dust. They are the principal cause of loss of yield in *ethanol fermentation*s. Otherwise referred to as lactic acid bacteria, they are generally *Gram-positive* and controllable with *penicillin* and certain other *antibiotics*.

Lactoside™ A combination of antibiotics formulated to take advantage of synergies in increasing the spectrum of activity against lactic and acetic acid-producing bacteria.

Lactose The principal *sugar* in milk and cheese *whey*. It may be fermented by suitable *yeast*s to *ethanol*. It is a *disaccharide* readily *hydrolysed* to its two components, *glucose* and *galactose*. Lactose has the formula $C_{12}H_{22}O_{11}$.

Lag phase Applied to *yeast propagation*, lag phase refers to the initial period in which the *yeast inoculum* becomes adapted to the *mash* prior to the rapid increase in cell numbers referred to as the *logarithmic phase*.

Latent heat The quantity of energy absorbed or released when a substance undergoes a change of state, i.e., from a solid to a liquid (melting) or from a liquid to a *vapor*, or vice versa. No change in temperature is involved. For example, water requires a large amount of latent heat (measured in BTUs or calories) to convert from the liquid to vapor state (steam) at 100°C, while steam releases the latent heat again on condensing back to the liquid state.

Lead phase-out The reduction in the amount of lead (normally in the form of *tetraethyl lead*) that could be used as an *octane enhancer* in leaded *gasoline* in the US. The reduction, which went from 1.1 grams per gallon prior to July 1, 1985 to less than 0.1 grams per gallon after January 1, 1986 caused an increase in demand for alternative octane enhancers such as *ethanol* and *MTBE*.

Light whisky Defined by the *BATF* as *whisky* produced at more than 160^0 *proof* and stored in used or uncharred oak *barrels*.

Lignin A *polymeric*, non-*carbohydrate* constituent of wood that functions as a support and binder for *cellulose* fibers. It may comprise 15-30% of wood and can only be separated from the cellulose and *hemicellulose* components by chemical reaction at high temperatures. Its presence in wood is a major barrier to the *hydrolysis* of cellulose to *sugars* for *fermentation* purposes.

Lignocellulose Woody materials made up largely of *lignin*, *cellulose* and *hemicelluloses*. The chemical bonding between the constituents renders it resistant to *hydrolysis*.

Lime A white alkaline powder composed of calcium oxide. It is added to grain *mash* to adjust the *pH* and to provide calcium ions in order to prevent the inactivation of the α-*amylase enzyme molecule* by the loss of its essential calcium atom.

Liquefaction Conversion of a solid substance to the liquid state. In reference to *starch*, it is the stage in the *cooking* and *saccharification* process in which *gelatinized starch* is partially *hydrolyzed* by α-*amylase* (or occasionally by an acid) to give soluble *dextrins*. This converts the *starch mash* into a free-flowing liquid, cutting viscosity.

Liqueurs and cordials Defined by the *BATF* as 'products obtained by mixing or redistilling distilled spirits with or over fruits, flowers, plants or pure juices therefrom, or any other natural flavoring materials, or with extracts derived from infusions, percolation or maceration of such materials, and containing *sugar, dextrose* or levulose, or a combination thereof, in an amount not less than 2.5% by weight of the finished product'.

Liter A metric measurement of volume defined as the equivalent of 1000 cubic centimeters or 0.2642 US gallons.

Logarithmic phase Applied to *yeast propagation*, it refers to the period in which cell numbers increase at an exponential rate after the initial *lag phase*.

Low boilers In reference to *ethanol distillation*, the term is applied to the *congeners*, or *fermentation by-products*, which boil at a lower temperature than *ethanol*. More commonly referred to as *heads*, these compounds are principally *aldehydes* and *methanol*.

LPA Abbreviation for *liters* of pure *alcohol*.

M

Macromolecule A giant *molecule* in which there is a large number of one or more relatively simple structural units or *monomers*.

Malt Barley grains that have been steeped in water and then allowed to germinate. The germination is normally halted by drying the grains when the sprouts are about the same length as the grains. At this stage, the malt (or 'malted barley') contains considerable amounts of α- *and ß-amylase enzyme*s that *saccharify* the barley *starch* and other additional *starch* in a *mash* to yield *fermentable sugars*. (In Scotland, the drying may be done by exposing the malt to a flow of peat smoke to impart a smoky odor to the malt). Malt is used in *whisky* production, where it also contributes to product flavor. In *fuel ethanol* production the necessary saccharifying *enzyme*s are normally derived from microbial sources.

Malt whisky In the US, malt whisky is defined by the *BATF* as a *whisky* produced at less than 160^0 proof from a fermented *mash* containing at least 51% malted barley, and stored at under 125^0 proof in charred, new oak *barrels*. In Scotland, malt whiskies are made from a 100% malted barley mash and may be aged in previously-used oak barrels. Malt whiskies may be mixed with *grain whiskies* to impart much of the characteristic flavor of blended Scotch whisky.

Maltase An *enzyme* capable of *hydrolyzing maltose sugar molecule*s into their two component *glucose* units. Occasionally the name is loosely applied to the *amyloglucosidase enzyme*, which *hydrolyzes polysaccharides* to *glucose*.

Maltose A *fermentable sugar* which is a *dimer* (or *disaccharide*) of *glucose* in that it is comprised of two linked *glucose* units. It is the normal end product of *starch saccharification* by the *ß-amylase enzyme* in *malt*.

Manioc See *cassava*.

Mannose A fermentable 6-carbon *sugar* (or *hexose*) which is an *isomer* of *glucose*. Chemical formula $C_6H_{12}O_6$. It is a constituent of *hemicellulose*.

Mash A mixture of milled grain or other fermentable *carbohydrate* in water used in the production of *ethanol*. The term may be used at any stage from the initial mixing of the *feedstock* in water prior to any cooking and *saccharification* through to the completion of *fermentation*, when it becomes referred to as *beer*.

Mash bill The percentages of different types of grains used in the preparation of a *mash* in *beverage alcohol* production. For example, a typical bourbon whisky mash bill may consist of 65% corn, 25% rye and 10% barley *malt*.

McCabe-Thiele diagram A graphic method for calculation of the number of *theoretical plates (or trays)* required in a *distillation column* to achieve a desired separation of two components.

Meal The floury or granular product resulting from milling or grinding of cereal grains.

Mechanical vapor recompression (MVR) A method used in *evaporation* in which the water vapor leaving the *evaporator* is recompressed and recycled to heat the same vessel. This means that mechanical energy is used rather than heat energy. The recompressor may be operated by electricity or by a steam turbine (if high pressure steam is available and there is a demand elsewhere for the low pressure turbine exhaust steam). Mechanical vapor recompression can greatly increase the steam usage efficiency, but the capital costs for equipment are also greater.

Metabolism The chemical processes in living cells by which energy is derived for vital processes, growth and activities.

Methane A colorless, odorless, tasteless, readily-combustible, asphyxiant, lighter-than-air gas. The first member of the paraffin (or alkane) series of *hydrocarbons*, it has a formula CH_4. It occurs in high proportions in natural gas, and is produced by decaying vegetation and other *organic* matter as in landfills and marshes. The methane produced in sealed landfills or from the *anaerobic digestion* of *thin stillage* and other wastewaters is used for boiler fuel in some *ethanol* plants.

Methane digester or methanator (or anaerobic digester) A system of tanks used for treatment of *organic* waste streams such as *thin stillag*e and evaporator condensate. The system is initially inoculated with a suitable culture of *methane*-producing *bacteria* and operates on a continuous basis to generate methane for use as a boiler fuel. Chemical oxygen demand of the water streams are reduced by this fermentation system.

Methanol (or methyl alcohol) A colorless poisonous liquid with essentially no odor and very little taste. It is the simplest *alcohol* and has the formula CH_3OH. It boils at 64.7°C. It is miscible with water and most *organic* liquids, including *gasoline*. It is extremely flammable, burning with a nearly invisible blue flame. It is a *congene*r of *ethanol fermentation*s. Having a lower *boiling point* than *ethanol*, it tends to be a major component of the *heads* stream on *distillation*. Due to its miscibility with *benzene*, its presence in a hydrous *ethanol* feed may reduce the efficiency of *dehydration* processes where benzene is used as an *entrainer*. *Methanol* is produced commercially by the catalyzed reaction of hydrogen and carbon monoxide. It was formerly derived from the destructive distillation of wood, which caused it to be known as *wood alcohol*. *Methanol* may be blended with *gasoline*, but requires a *co-solvent* such as *ethanol* or a *higher alcohol* to maintain it in *solution*. (See *Demethylizing column*).

Methyl tertiary butyl ether (MTBE) A colorless, flammable, liquid *oxygenated hydrocarbon*. Chemical formula $(CH_3)_3COCH_3$. It contains 18.15% oxygen and has a *boiling point* of 55.2°C. It is produced by reacting *methanol* with *isobutylene*. It is used as an *octane enhancer* in *gasoline*, but is being phased out due to pollution.

MFGE Abbreviation for *motor fuel grade ethanol*.

Microorganism A collective term for microscopic organisms including *bacteria*, *yeast*s, viruses, algae and protozoa.

Milo (or millet, or grain sorghum) Much smaller than corn, this cereal grain has a similar *starch* content and yields almost the same amount of *ethanol* per *bushel* on *fermentation*. Milo is more drought-resistant than corn and is frequently grown in areas unsuited to corn. Milo *fermentation*s may tend to foam more than corn fermentations and will produce a characteristic surface crust if not agitated.

Molar solution A solution of salts or other substances in which one *molecular weight* of the substance in grams (one *mole*) is dissolved in enough *solvent* to make up to one liter. Molarity (or molar concentration) is a measure of moles per *liter* of a solution and is indicated by the letter M as in 1M, 10M, etc.

Molasses The thick liquid remaining after *sucrose* has been removed from the mother liquor (of clarified concentrated cane or beet juice), in *sugar* manufacture. *Blackstrap* molasses is the syrup from which no more sugar may be removed economically. It has usually been subjected to at least three *evaporating* and *centrifuging* cycles to remove the crystalline sucrose. Its analysis varies considerably, depending on many factors including sugar mill equipment and operational efficiency; but it may contain approximately 45-60% *fermentable sugars* by weight and approximately 10% ash (or minerals). It is commonly used as an *ethanol feedstock* when prices are favorable. *High test molasses (HTM)* is not a true molasses as it is the mother liquor from which no crystalline sugar has been removed by centrifugation but which has been treated with acid to reduce crystallization. It may contain approximately 80% sugars by weight and is very low in ash. Typically HTM is only produced in years when the sugar price does not justify its recovery. It may be used as an *ethanol* feedstock when prices are favorable, and has the advantage over blackstrap of causing less *distillation column scaling*. However, it requires more nutrients for *fermentation*. (See also *citrus molasses)*.

Mole A gram mole is the quantity of a chemical compound or element equal to its *molecular weight* in grams. By this definition, moles of different compounds or elements contain the same number of *molecule*s. Thus in the *vapor* state, as ideal gases, moles of different compounds occupy the same volume at the same temperature and pressure. This leads *distillation* equipment designers to base their calculations on the mole percentages of different compounds in a mixture to be separated, such as *ethanol* and water, rather than the percentages by weight or volume.

Molecule The smallest unit into which a pure substance can be divided and still retain the composition and chemical properties of the substance. For example the formula for water is H_2O; and the smallest unit recognizable as water is the single molecule of two atoms of hydrogen linked to one atom of oxygen.

Molecular sieve A microporous substance composed of materials such as crystalline aluminosilicates belonging to a class known as *zeolites*. The size of the pores in the substance may vary with its chemical structure, being generally in the range of 3 to 10 *Angstrom* units in diameter. With material having a very precise pore size, it is possible to separate smaller *molecule*s from larger ones by a sieving action. For example, in *ethanol dehydration* with a *potassium aluminosilicate* material prepared with pores of a diameter of 3 Å units, water *molecule*s with a diameter of 2.5 Å may be retained by adsorption within the pores while *ethanol molecule*s of a diameter of 4 Å cannot enter and therefore flow around the material.

The term molecular sieve is frequently used loosely to describe the entire *ethanol* dehydration apparatus holding the beads of sieve material and includes the equipment and controls necessary to regenerate them when saturated with water.

Molecular weight The sum of the *atomic weights* of the atoms in a *molecule*. For example, water *molecule*s are composed of two atoms of hydrogen (atomic weight of one) and one atom of oxygen (atomic weight of 16) to give a total molecular weight of 18.

MON Abbreviation for *motor octane number*.

Monomer A single *molecule* of a substance of relatively low *molecular weight* and simple structure, which is capable of conversion to *polymers* by combination with other identical or similar *molecule*s. For example, *glucose* is a monomer, which by interlinking of *molecule*s can be built into large polymers such as the *amylose* and *amylopectin* contained in *starch*.

Monosaccharide A *sugar monomer*. Examples are *glucose*, *fructose* and *galactose*. These are the simplest forms of sugars, which are more readily fermented by *yeast*s than their *polymers* (or *polysaccharides*).

Mother yeasting A system of *yeast propagation* frequently used for *molasses fermentation*s in which the propagator is not emptied entirely when inoculating a *fermentor* and the portion retained is used for starting another *yeast* propagation cycle. By adding acid to maintain a low *pH* in the propagator it may be possible to repeat the process for numerous cycles without excessive *bacterial contamination*.

Motor fuel grade ethanol (MFGE) Refers to *anhydrous ethanol* prior to *denaturation* to *fuel ethanol*. The term is used to distinguish it from the various grades of *industrial* and *beverage alcohol*. MFGE is relatively crude, with considerable impurities, but conforms to legal *anhydrous* standards. It has not undergone the *rectification* required to make it suitable for industrial or beverage uses.

Motor octane number (MON) One of two methods commonly used to assess the *octane rating* of an automobile fuel. (See *Octane rating*).

MSW Abbreviation for *municipal solid waste.*

MTBE Abbreviation for methyl tertiary butyl ether.

Multiple effect evaporator A system comprising a series of interlinked *evaporator* vessels (or '*effects*') operating under increasing degrees of vacuum. As liquids boil at lower temperatures with increasing vacuum, it is possible to use the *latent heat* of the *vapors* from one vessel to heat the next (and so on through a series of up to about six vessels under increasing vacuums) with steam or other external source of heat only being introduced to the first vessel. This allows considerable economy in energy consumption. Multiple effect evaporators are commonly used for concentrating *thin stillage* solids into a syrup.

Municipal solid waste (MSW) Regular city trash or garbage. There have been numerous proposals and some pilot-scale attempts to produce *ethanol* from MSW by *hydrolyzing* and *fermenting* the *cellulosics* contained in the material. Such efforts have, however, generally run into problems due to the normal wide variability of MSW.

MVR Abbreviation for *mechanical vapor recompression.*

Mycotoxin A toxic substance produced by molds.

N

Near infrared spectroscopy (NIR) NIR analysis employs light beyond the visible spectrum to provide analysis of organic substances without the need for sample destruction. Its advantages to process management are virtually instantaneous results versus the hours or days needed with wet chemistry or chromatography. Applications include analysis of incoming grains, profiling fermentors, distillate analysis, dry house operations, and finished products.

Net energy balance The amount of energy available from a fuel by combustion, less the amount of energy taken for its production. In the early 1980s a lot of emphasis was placed on the need for a net energy balance in the production of *fuel ethanol* to be used as a *gasoline extender*. The emphasis was later reduced when it became appreciated that a) much of the energy used in *ethanol* production may come from low quality sources such as coal, wood or bunker-C oil, which cannot be used as automobile fuels, and b) that *ethanol* has a value as an *octane enhancer* and oxygenate and not just as a *gasoline* extender. In recent work the net energy balance in corn to ethanol conversion is 1.3 t.

Neutral spirit Defined by the *BATF* as 'distilled spirits produced from any material at or above 190^0 *proof*'. In practice, neutral spirit is purified, odorless, tasteless and colorless *ethanol* produced by *distillation* and *rectification* techniques that remove any significant amount of congeners. It is used in the production of beverages such as *vodka, gin, cordials* and cream *liqueurs*.

Nitrogen oxides Air-polluting gases contained in automobile emissions, which are regulated by the *EPA*. They comprise colorless nitrous oxide (N_2O) (otherwise know as dinitrogen monoxide or as the anaesthetic 'laughing gas'), colorless nitric oxide (NO) and the reddish-brown nitrogen dioxide (NO_2). Nitric oxide is very unstable and on exposure to air it is readily converted to nitrogen dioxide, which has an irritating odor and is very poisonous. It contributes to the brownish layer in the atmospheric pollution over some metropolitan areas. Other nitrogen oxides of less significance are nitrogen tetroxide (N_2O_4) and nitrogen pentoxide (N_2O_5). Nitrogen oxides are sometimes collectively referred to as NO_x (or Nox) where x represents any proportion of oxygen to nitrogen.

Normal solution A *solution* containing one equivalent weight of a dissolved substance per liter. One volume of a normal acid will neutralize an equal volume of a normal alkali (or vice versa). This principle is used in measuring the acidity in *fermentation*s by *titration* with a standardized alkali. The 'normality' of solutions is indicated by the letter N, as in 0.1N, 2N, etc.

O

Occupational Safety and Health Administration (OSHA) An agency of the US Department of Labor with the mission of developing and promulgating occupational safety and health standards, developing and issuing regulations and conducting inspections and investigations to ensure their compliance.

Octane A flammable liquid *hydrocarbon* of chemical formula C_8H_{18} found in *petroleum.* One of the eighteen *isomers* of octane, 2,2,4-trimethylpentane is used as a standard in assessing the *octane rating* of fuels.

Octane enhancer Any substance such as *ethanol*, *methanol MTBE, ETBE, benzene, toluene, xylene,* etc., that will raise the *octane rating* when blended with *gasoline*.

Octane rating (or octane number) A laboratory assessment of a fuel's ability to resist self-ignition or 'knock' during combustion in a spark-ignition engine. A standardized-design, single-cylinder, four-stroke engine with a variable compression ratio is used to compare the knock resistance of a given fuel with that of reference fuels composed of varying proportions of two pure *hydrocarbons*. One component is the *octane isomer* 2,2,4-trimethylpentane (sometimes referred to as isooctane), which has a high resistance to knock and is given the arbitrary rating of 100. The other component, heptane, has a very low knock resistance and is given the base rating of zero. For fuels with a rating higher than 100 octane, the rating is obtained by determining how much *tetraethyl lead* needs to be added to pure isooctane to match its knock resistance.

The engine knock tests are performed under two sets of operating conditions, the so-called 'motor' or M method, and the 'research' or R method. The average of the two results is taken to be a good indicator of a fuel's performance in a typical automobile on the road. Hence, the use of the *(R+M)÷2* octane rating on labels on service pumps.

OFA Abbreviation for *Oxygenated Fuels Association.*

Oligomer A *macromolecule* formed by the chemical union of two, three or four identical units known as *monomers*. The oligomers of two units are *dimers*, three units are *trimers* and four units are *tetramers*. (The prefix oligo- means 'few').

Oligosaccharide Short-chain *polymers* of simple *sugars* (or *monosaccharides*) generally considered to cover the range of 2-8 units. Short *dextrins* produced by *hydrolysis* of *starch* are included in this category.

OPIS Abbreviation for *Oil Price Information Service.*

Organic Adjective for chemical compounds containing carbon and hydrogen with or without oxygen, nitrogen or other elements.

Organoleptic testing The quality control process of checking samples of alcoholic products on the basis of odor and taste. It is normally performed by comparing samples of new production with older samples of acceptable quality that have been designated as standards.

Orifice meter An instrument used to measure fluid flow by recording the differential pressure across a restriction (or 'orifice plate') placed in the flow stream.

OSHA Abbreviation for *Occupational Safety and Health Administration.*

Overhead vapors The *vapors* emerging from the top of a *distillation column* that are conducted to a *condenser* system.

Oxygenated fuels Literally meaning any fuel substance containing oxygen, the term is commonly taken to cover *gasoline*-based fuels containing such oxygen-bearing compounds as *ethanol*, *methanol, MTBE, ETBE*, etc. Oxygenated fuel tends to give a more complete combustion of its carbon to *carbon dioxide* (rather than *monoxide*) and thereby to reduce air pollution from exhaust emissions.

P

Packed distillation column A column filled with a packing of ceramic, metal or other material designed to increase the surface area for contact between liquids and *vapors.*

PADD Abbreviation for *Petroleum Administration for Defense District.*

Pasteurization A method devised by Pasteur to partially sterilize a fluid by heating to a specific temperature for a specific length of time. Pasteurization does not greatly change chemical composition, but destroys undesirable *bacterial contaminants*. The practice may be applied to *molasses* or other liquid *fermentation feedstocks* to reduce the initial level of *bacterial contamination.*

Patent A certificate or grant by a government of an exclusive right with respect to an invention for a limited period of time. In the US the effective period is normally 17 years from the date of granting. Patents may cover processes, machines or other apparatus, methods of manufacture, composition of materials and designs. Patents are published and freely available and are required to provide sufficient information on an invention so that anyone 'reasonably versed in the art' would be able to implement it. Thus, patents can be very useful sources of technical information. Regardless of what may be stated in the title, summary or introduction, the legal essence of a patent is in the very precisely and narrowly-defined claims appearing at the end of the text. Generally, the patent examiner does not revise the summary of a patent to comply with the claims that are finally approved; so that the summaries published in various journals can be quite misleading as to the scope of the respective patents.

Pentane A colorless, highly-flammable *hydrocarbon* liquid with a pleasant odor. Chemical formula C_5H_{12}, *boiling point* 37.1°C, freezing point -129.7°C. It is used as an *entrainer* for the *dehydration* of *ethanol* by *azeotropic distillation*. It presents a severe explosion risk at low concentrations in air.

Pentanol See *amyl alcohol.*

Pentose General term for *sugars* with five carbon atoms per *molecule* such as *xylose* and *arabinose,* which are constituents of *hemicellulose*. Pentoses are not fermented by normal *strains* of distillers *yeast*s.

Permanganate (or Barbet) time A laboratory test used for assessing the quality of samples of *industrial* or *beverage alcohol*. It is the time required for an alcohol sample to decolorize a standard potassium permanganate solution. The time is an indication of the reducing (deoxidizing) power of the sample, and is considered to be a crude measure of the presence of congeners.

Petroleum A naturally-occurring complex *hydrocarbon*, which may be liquid (crude oil), gaseous (natural gas), solid (asphalt, tar or bitumen) or a combination of these forms.

Petroleum Administration for Defense District (PADD) A designation of regions in the US for the purposes of the *Department of Energy*'s presentation of statistics on the usage of *petroleum*-based fuels including diesel fuel, *gasoline* and *gasoline-ethanol* blends. The term originated in World War II as 'Petroleum Administration for War Districts'. The boundary lines were drawn to reflect the natural logistical regions of the petroleum economy. Thus, the country is divided into 5 PADDs: No. 1 (east coast) includes primarily the area receiving most of its supply of crude oil by tanker, No. 2 (midwest) and No. 3 (Gulf coast) are defined in light of normal transport and supply operations, No. 4 (Rocky Mountains) is largely self-contained, No. 5 (west coast) is naturally isolated from the rest of the country.

PG Abbreviation for *proof gallon.*

pH A value measuring the acidity or alkalinity of an *aqueous solution*. Defined as the logarithm of the reciprocal of the hydrogen ion concentration. Pure water at room temperature has a pH of 7.0. Solutions with a pH of less than 7 are acidic, and greater than 7 are alkaline. As the pH scale is logarithmic, a solution with a pH of 5 has 10 times the acidity of a solution of pH 6. Control of pH is important in *ethanol* production both for obtaining optimal *enzymatic* activity and in controlling the growth of *bacterial contaminants*.

Phase separation The phenomenon of a separation of a liquid (or *vapor*) into two or more physically distinct and mechanically separable portions or layers. It is used to advantage in the *dehydration* of *ethanol* by *azeotropic distillation* with an *entrainer* such as *benzene*. When the *overhead vapors* are condensed, the liquid separates into two distinct layers or phases. The upper phase contains most of the benzene with some *ethanol* and a little water, while the lower contains most of the water with some *ethanol* and a little benzene. The lower layer is removed and redistilled in an entrainer recovery column to recover the benzene and *ethanol* and to discharge the bulk of the water.

Plate (or tray) A contacting device placed horizontally at intervals within a *distillation column*. Plates may be simple perforated discs with or without *downcomers* as in the *sieve plate* and the *dual flow plate*, or they may have *bubble caps, tunnel caps* or various types of floating valves to improve the contact between the rising *vapor* and descending liquid. Sieve plates and tunnel caps are the most common form of plates used in *ethanol* production facilities.

Plate (or tray) distillation column A *distillation column* with horizontally arranged contacting *plates* located at intervals up the column; as compared to a *packed column,* which is filled randomly with contacting devices.

Polymer A *macromolecule* formed by the chemical union of identical combining units known as *monomers*. While generally referring to a combination of at least five monomers, in many cases the number of monomers is quite large, such as the average of 3,500 *glucose* monomers in *cellulose*.

Polysaccharide A *polymer* composed of numerous *sugar monomers* or *monosaccharides*. Examples are *cellulose* and the *amylose* and *amylopectin* in *starch*. For *fermentation* purposes, polysaccharides must be subjected to hydrolysis to yield their component fermentable monosaccharides.

Positive displacement pump A pump in which the displacement is accomplished by mechanically moving a volume of fluid from the pump suction to the discharge with no fluid slippage within the pump - no matter how high the discharge pressure. With every stroke or rotation the same volume of fluid is pumped. Positive displacement pumps may take various forms such as reciprocating piston pumps, gear pumps, lobe pumps and diaphragm pumps. Generally, they are used to move relatively low volumes of fluid at high pressures. As they force a positive displacement, they may not be used in systems where the output may be throttled or closed off without incorporating a pressure relief or recycle system. Positive displacement pumps are frequently used for moving *molasses* and *stillage* syrups, yeast slurries and fuel oil. They are also effective for metering since every rotation or stroke moves a precise volume from the suction side to the discharge side regardless of the pressure required.

Potassium aluminosilicate A zeolite. (See *molecular sieve).*

Pot still A simple *batch distillation* unit used for the production of heavily-flavored *distillates* for beverage use. It consists of a tank (heated either by an internal steam coil or by an external fire) and an *overhead vapor* pipe leading to a *condenser.* It may be used in the production of heavily-flavored *rums* and *whiskies*.

Pre-fermentor See *yeast* propagator.

Proof A measure of the *absolute ethanol* content of a *distillate* containing *ethanol* and water. In the US system, each degree of proof is equal to 0.5% *ethanol* by volume, so that absolute *ethanol* is 200^0 proof. In the Imperial system 100 proof is equal to 57.06% *ethanol* by volume, or 48.24% by weight, while absolute *ethanol* is 75.25 over proof, or 175.25 proof.

Proof gallon (PG) The volume of a liquid that contains the equivalent of one gallon of *ethanol* at 100^0 *proof.*

Proof tables Books of tables compiled by the *BATF* and other organizations for the calculation of *proof* of *ethanol*-containing liquids with adjustments for varying temperatures.

Propagation The process of increasing numbers of organisms by natural reproduction. In the case of *yeast*, propagation occurs by budding to form new cells in both aerobic and anaerobic conditions. Yeast companies grow yeast under aerobic conditions with high yield. Fuel alcohol propagation of yeast is normally under anaerobic conditions (even with air supplied) due to the presence of high amounts of sugar. (See *yeast propagator).*

Propanol (or propyl alcohol) A minor constituent of *fusel oil*. Chemical formula C_3H_7OH. It exists as either of two *isomers*. Both are colorless, toxic, flammable liquids with odors similar to that of *ethanol*.

Protein Any of a class of high *molecular weight polymer* compounds composed of a variety of linked amino acids. Proteins are an essential ingredient in the diets of animals and humans. *Yeast* dry matter is approximately 50% protein. Corn *DDG* generally contains over 27% crude protein.

PSI Abbreviation for pounds per square inch. Atmospheric pressure is 14.7 psi, 1 bar is 14.5 psi.

PSIG Abbreviation for pounds per square inch gauge.

R

Rack price Used in reference to *gasoline* and *fuel ethanol*, it is the wholesale price at the tank-truck loading terminal exclusive of any federal, state or local taxes.

Reboiler A device for supplying heat to a *distillation column* without introducing live steam. It generally consists of a shell-and-tube *heat exchanger* connected to the base of the column with liquid from the column entering inside the tubes to be heated indirectly by steam on the shell side.

Receiver A tank into which new *distillates* flow from the *still* for verifying *proof*, quantity and quality before transfer to shipping or storage tanks. Where the tank is sealed or has locks on the valves to meet government excise regulations for preventing unlawful access, it may be referred to as a 'closed receiver'.

Recoopered barrels Used wooden *barrels* that have been repaired to replace any cracked staves or heads to be suitable for reuse in the aging of alcoholic beverages.

Rectification The process of concentrating and purifying *ethanol* or other materials in a *rectifying column*. The process may be operated simultaneously with *beer distillation* with the rectifying column directly connected to a *beer stripping column*. In *beverage ethanol* production the rectification process may involve concentration and removal of *fusel oil, esters* and *heads*. In *fuel ethanol* production the process commonly only involves concentration and the removal of fusel oil. In some instances, where *benzene* is to be used as an *entrainer* in the subsequent *dehydration* process, the heads may also be removed in the rectification process.

Rectifying column (rectifier, rectification column or rectifying section) The portion of a *distillation column* above the *feed tray* in which rising *vapor* is enriched by interaction with a countercurrent descending stream of condensed vapor. In the case of *ethanol* production, the rectifying column may have valves at draw-off points on various trays to allow the removal of accumulated *fusel oil* and other *congeners*. In some instances the *ethanol* product is withdrawn from the *reflux* of the *overhead vapor condensate*, but in others the product may be drawn from one of the upper trays to permit the *heads* or *low boilers* to be purged from the reflux. (See *Concentrating column).*

Reflux The portion of the condensed *overhead vapors* returned to a *distillation column* to maintain the liquid-vapor equilibrium.

Reflux ratio The ratio of the amount of *condensate refluxed* to the amount withdrawn as product. Generally, the higher the reflux ratio the greater the degree of separation of the components in a *distillation* system.

Refractometer An instrument used to measure the refractive index of liquids and liquid solutions. Refractometers can be calibrated in concentration of a solute instead of refractive index. In the ethanol industry refractometers are calibrated as degrees Brix or degrees Balling.

Reid vapor pressure (Rvp) A measure of the *vapor pressure* in pounds per square inch of a sample of *gasoline* at 100°F. It is an indication of the *volatility* of a *gasoline*. The blending of *ethanol* with *gasoline* tends to increase Rvp while the blending of *MTBE,* and more particularly *ETBE,* tends to reduce Rvp.

Renewable Fuels Association (RFA) The Washington DC-based trade association for the US *fuel ethanol* industry. Its membership includes companies involved in the production, blending and marketing of *ethanol*-blended fuels.

Research octane number (RON) See *Octane rating*.

Reverse osmosis A technique used in water purification and wastewater and *stillage* treatment in which pressure is applied to the liquid in a suitable apparatus to force pure water through a membrane that does not allow the passage of dissolved ions.

RFA Abbreviation for *Renewable Fuels Association*.

Rhizozyme™ Saccharifying enzyme complex produced in surface culture fermentation. As it is cultured on wheat bran, a range of carbohydrases are produced in addition to glucoamylase, which results in more extensive liberation of fermentable sugars from grain sources.

(R+M)÷2 The formula for calculating *octane rating*.

Roller mill A mill for crushing or grinding grain or other solid material by passing it between two, and as many as six, steel rollers. The rollers may be smooth, or serrated to shear the grain, and they may turn at differing speeds to increase the abrasion. Roller mills are suitable for small grains such as wheat, but do not perform as well as a *hammer mill* on corn.

RON Abbreviation for *research octane number*.

Rotameter A *flow meter* with a float and a variable area flow tube.

Rum Defined by the *BATF* as 'an alcoholic *distillate* from the fermented juice of sugarcane, sugarcane syrup, sugarcane *molasses*, or other sugarcane by-products, produced at less than 190^0 *proof*, in such a manner that the distillate possesses the taste, aroma and characteristics generally attributed to rum'. Unlike the specifications for *whiskies*, the *BATF* does not require that rum be aged in oak *barrels*. British regulations specify that rum be produced 'from sugarcane products in sugarcane-growing countries'.

Rvp Abbreviation for *Reid vapor pressure*.

Rye whisky Defined by the *BATF* as *whisky* produced at not more than 160^0 *proof*, from a fermented *mash* of not less that 51% rye and stored at not more than 125^0 proof in charred, new oak *barrels*.

S

Saccharification The process of converting a complex *carbohydrate* such as *starch* or *cellulose* into *fermentable sugars* such as *glucose* or *maltose*. It is essentially a *hydrolysis*. The process may be accomplished by the use of *enzyme*s or acids.

Sacc' tank (or saccharification tank) A vessel where cooked *mash* is held for the *saccharification* process. Sacc' tanks are generally going out of use with the adoption of *simultaneous saccharification and fermentation* in the *fermentor*.

Saccharomyces A genus of unicellular *yeast*s of the family Saccharomycetaceae distinguished by the general absence of mycelium and by their facility to reproduce asexually by budding. This genus includes the species *Saccharomyces cerevisiae*, which is the *yeast* most commonly used by bakers, brewers, distillers and wine producers.

Sanitizing In processing systems, sanitizing is reducing the population of viable organisms in the equipment to a level that is acceptable for the process.

Scale A deposit formed on the surface of equipment due to formation of insoluble inorganic compounds.

Scale inhibitor See *Antiscalant.*

Scaling The precipitation of salts in a *distillation column* or *heat exchanger* which if uncontrolled may reduce capacity and eventually block the equipment and make it unusable. Scaling occurs because some salts such as calcium sulfate and oxalate are less soluble at high temperatures and/or in the presence of *ethanol*. Scaling problems are more common in *molasses beer* distillation, but may occur in grain beer distillation when conducted under pressure (i.e., at high temperatures).

Scrubber A device for the removal of entrained liquid droplets in a gas stream. Scrubbers may be used to recover *ethanol* from the *carbon dioxide* vented from *fermentors.*

SDA Abbreviation for *specially denatured alcohol.*

SG Abbreviation for *specific gravity*.

Shooked barrel (or shook) A used *bourbon whisky barrel* that has been dismantled to reduce the space requirements for transportation.

Sieve analysis A laboratory test made on grain *meal* to check that the milling process is being conducted correctly. The meal is added to the top of a stack of sieves with increasingly fine mesh sizes descending downwards. The sieve stack is vibrated for a standard time period and the weight percentage retained on each screen is determined. With *hammer mills*, the sieve analysis will generally show that the meal gradually becomes more coarse as the hammers wear and need turning or replacement.

Sieve plate (or sieve tray) A *distillation column plate* with perforations of precise number and size such that the ascending *vapor* passes through the plate vertically to mix with the descending liquid held on the plate. A sieve plate differs from a *dual flow plate* in that it is designed for the descending liquid to overflow down a *downcomer*, instead of *weeping* through the perforations.

Simultaneous saccharification and fermentation (SSF) A procedure in which *saccharification* of a cooked (gelatinized and liquefied) *starch mash* occurs in the *fermentor* (by addition of glucoamylase) simultaneously with the commencement of *fermentation* (by *yeast*). This procedure is replacing the traditional process taken from the whisky industry in which there is a specific holding stage for saccharification with *malt* or microbial *enzyme*s (in a *sacc' tank*) before the mash goes to a fermentor.

Slurry tank The vessel in which grain *meal* is mixed into a slurry with water and enzyme before being pumped through a *continuous cooking* system.

Small Business Administration (SBA) A US federal government agency charged with encouraging and assisting the development of small businesses. It offers financial assistance such as direct loans, loan guarantees and direct participation loans for virtually any legitimate small business development. The SBA has assisted in the financing of many small *fuel ethanol* facilities.

Soil Any unwanted material that is left on a surface that needs to be clean.

Solute One or more substances dissolved in another substance (a *solvent*) to form a *solution*. The solute is uniformly dispersed in the solvent in the form of *molecule*s (as with *sugars*) or ions (as with salts).

Solution A uniformly dispersed mixture at the *molecular* or ionic level of one or more substances (the *solute*) in one or more other substances (the *solvent*). *Ethanol* and water form a liquid-liquid solution, while sugar and water form a solid-liquid solution.

Solvent A substance capable of dissolving another substance (a *solute*) to form a uniformly-dispersed mixture (*solution*) at the *molecular* or ionic level.

Specially denatured alcohol (SDA) The term used to describe *ethanol* denatured with any formulation of compounds selected from a list approved by the *BATF.* The *denaturant* renders the *ethanol* unfit for beverage purposes without impairing its usefulness for other applications.

Specific gravity (SG) The ratio of the density of a material to the density of a standard reference material such as water at a specific temperature. For example, the specific gravity of *ethanol* is quoted as 0.7893 at 20/4^{o}C. This means that a given volume of *ethanol* at 20^{o}C weighs 0.7893 times the weight of the same volume of water at 4^{o}C.

Spent sulfite liquor (SSL) See *sulfite waste liquor*.

Spirit whisky Defined by the *BATF* as a mixture of *neutral spirits* with at least 5% on a *proof gallon* basis of *whisky* or *straight whisky*.

SSF Abbreviation for *simultaneous saccharification and fermentation*. In the enzyme industry SSF also is used to indicate solid-state fermentation.

SSL Abbreviation for *spent sulfite liquor*. (See *sulfite waste liquor).*

Starch A mixture of two *carbohydrate polymers* (*amylose* and *amylopectin*), both of which are composed of *glucose monomers* linked by glycosidic bonds. *Starch* is a principal energy storage product of photosynthesis and is found in most roots, tubers and cereal grains. *Starch* may be subjected to *hydrolysis* (*saccharification*) to yield *dextrins* and *glucose*.

Sterilization The complete destruction of all organisms including viruses and spores.

Still An apparatus used in *distillation* comprising a vessel in which the liquid is *vaporized* by heat and a cooling device in which the *vapor* is condensed. It may take the form of a batch or continuously operable unit.

Stillage The mixture of non-fermentable (or non-fermented) dissolved solids, insoluble grain fines proteins, dead yeasts and water, which are the residues after removal of *ethanol* from a fermented *beer* by *distillation.* Stillage may be dried to recover the solid material (as *DDG* in the case of grain *feedstocks*).

Stoichiometric yield The theoretical yield of a product of a chemical reaction as calculated from the chemical reaction equation. For example, in the *Gay Lussac* equation for the *fermentation* of *glucose* to *ethanol* by *yeast*, $C_6H_{12}O_6 \rightarrow 2CO_2 + 2C_2H_5OH$, 100 parts (by weight) *glucose* should yield 48.89 parts *carbon dioxide* and 51.11 parts *ethanol.* Due to a variety of factors, this yield is not achieved in practice.

Stoichiometry The branch of chemistry that deals with the quantities of substances that enter into and are produced by chemical reactions.

Stover The dried stalks and leaves remaining from a crop, particularly corn, after the grain has been harvested. It is of interest as a potential source of *cellulose feedstock* for *ethanol* production.

Straight whisky Defined by the US Bureau of Alcohol, Tobacco and Firearms as a product conforming to the requirements for the *whisky* designation, which has been stored in charred, new oak *barrels* for a period of at least two years. The straight whisky definition may include mixtures of straight whiskies of the same grain type, produced at the same *distillery*, all of which are not less than four years old.

Strain See *Yeast strain.*

Stripping column (or stripping section) The portion of a *distillation column* below the *feed tray* in which the descending liquid is progressively depleted of its *volatile* components by the introduction of heat at the base.

Sub-octane blending The practice of blending *ethanol* with selected *gasoline* components that have *octane ratings* below the commercially or legally acceptable level. It takes advantage of the fact that *ethanol* can enhance the octane rating of the mixture to bring it to an acceptable level.

Sucrose Common table *sugar*, derived from beet or cane sources. It is a *disaccharide* comprising a *monomer* of *glucose* linked to a monomer of *fructose*. It has a chemical formula $C_{12}H_{22}O_{11}$. It is directly fermentable by common *yeast*s to produce *ethanol.*

Sugar Any of a class of water-soluble, simple *carbohydrate*, crystalline compounds that vary widely in sweetness including the *monosaccharides* and lower *oligosaccharides*. Sugars may be chemically reducing or non-reducing compounds and are typically optically active. Examples include the monosaccharides *glucose*, *fructose, mannose* and *xylose*, and the *disaccharides sucrose, maltose, lactose,* and the trisaccharides raffinose and maltotriose.

Sulfite waste liquor (SWL) An effluent produced in the sulfite pulping process used in some paper mills. It partly consists of a dilute solution of *sugars* produced by the *acid hydrolysis* of *cellulose*. It may be used as a *feedstock* for the production of *ethanol* by *fermentation* using selected *yeast* strains after stripping out the sulfite (or sulfur dioxide) with steam.

Supplementary column See *Demethylizing column.*

Sweet sorghum A plant of the species *Sorghum bicolor*, closely related to *milo or grain sorghum*, which is cultivated primarily for the sweet juice in its stem. It is widely used for cattle fodder and silage. The juice contains *sucrose*, which may be extracted by crushing with modified sugar mill equipment. The juice may be used in the fermentative production of *ethanol*. The plant has an advantage over sugarcane in that it can be grown over a much wider range of climatic regions.

SWL Abbreviation for *sulfite waste liquor.*

Synthetic ethanol *Ethanol* produced by any of several synthetic processes such as the catalytic hydration of ethylene, the sulfuric acid hydration of ethylene and the Fischer-Tropsch process, in which it is a major by-product of the synthesis of *methanol* by catalytically reacting *carbon dioxide* and hydrogen. Synthetic *ethanol* is chemically identical to *fermentation ethanol*, but does not qualify for US federal or state incentives for blending with *gasoline* and may not be used in the production of alcoholic beverages.

T

Tafia An unaged Caribbean or South American alcoholic beverage produced by *batch distillation* of *beers* obtained by the *fermentation* of sugarcane juice or *molasses*. It is similar to *aguardiente* and *rum.*

Tails See *high boilers.*

TBA Abbreviation for tertiary *butyl alcohol*. (See *butanol*).

Tequila Defined by the *BATF* as 'an alcoholic *distillate* from a fermented *mash* derived principally from *Agave tequilana Weber* ('blue' variety) with or without additional fermentable substances, distilled in such a manner that the distillate possesses the taste, aroma and characteristics normally attributed to tequila. It is a distinctive product of Mexico, manufactured in that country in compliance with the laws regulating the manufacture of tequila for consumption in that country'. Tequila may be matured in wooden containers to acquire a light gold color.

Ternary azeotrope An *azeotrope* or *constant boiling mixture* made up of three components. For example, a mixture of 74% volume *benzene*, 18.5% *ethanol* and 7.5% of water forms an azeotrope boiling at 64.9°C.

Tetramer A *macromolecule* produced by linking four identical units known as *monomers*. (See *oligomer)*.

Tetraethyl lead (or lead tetraethyl) An organometallic compound used as an *octane enhancer* in *gasoline*. Its use is regulated by the *EPA* to control air pollution. If added to *gasoline* on its own, tetraethyl lead would leave combustion residues of lead and lead oxide on engine cylinder walls. To prevent accumulation of these deposits, ethylene dibromide and dichloride are also added to convert the lead to *volatile* halides before release into the atmosphere.

Theoretical plate (or tray) A *distillation column plate* or *tray* that produces perfect *distillation* i.e. it would yield the same difference in composition between liquid and *vapor* as that normally existing between the liquid and the *vapor* when in equilibrium. A distillation column giving the same separation as 10 simple theoretical distillations is said to have 10 theoretical plates.

Theoretical yield See *stoichiometric yield*.

Thermal efficiency The ratio of the energy output of a process to the energy input.

Thermal vapor recompression A method using the same recompression and recirculation principles as in *mechanical vapor recompression* (to enhance the efficiency of *evaporation)* except that recompression is performed by passing steam through a venturi jet on the vapor line.

Thermophilic Literally meaning 'heat loving', it refers to *microorganisms* such as *bacteria* that thrive at warm temperatures, generally in the range of 35-60°C. Lactobacillus is an example of a thermophilic bacterium that multiplies rapidly at around 55°C. Appreciation of this fact has been a reason why the use of *sacc' tanks* has fallen into disfavor.

Thermosacc™ Stress-tolerant (heat and ethanol) strain of *Saccharomyces cerevisiae* used in ethanol production.

Thin stillage The liquid portion of *stillage* separated from the solids by screening or *centrifuging*. It contains some yeast, suspended fine particles and dissolved material. It is normally sent to an *evaporator* to be concentrated to a thick syrup and then dried with the solids portion to give *DDGS*.

Titration A method of volumetrically determining the concentration of a substance in *solution* by adding a standard solution of known volume and strength until the reaction between the two is completed, usually as indicated by a change in color due to an added chemical indicator. For instance, it is common practice to perform a titration with 0.1 *Normal* sodium hydroxide solution using phenol-phthalein as indicator to determine the acidity of a *mash* or *beer*.

Toluene An aromatic *hydrocarbon* compound that can be used as an *octane enhancer* in *gasoline*. It is a colorless, flammable liquid with a benzene-like odor. Chemical formula

$C_6H_5CH_3$. It boils at 110.7°C and freezes at -94.5°C. It is derived from the catalytic reforming of *gasoline* or the *distillation* of coal tar light oil. It is known to be carcinogenic.

Total sugars as invert (TSAI) A simple crude analytical measure of reducing *sugars* in *molasses*.

Transglucosidase An *enzyme* with the opposite effect of *amyloglucosidase* in that it brings about a *polymerization* of *glucose* into *polysaccharides*. It may be present as an impurity in commercial *enzyme* preparations.

Trimer A *macromolecule* produced by the linking three identical units known as *monomers*. (See *oligomer).*

Tray See *plate*.

TSAI Abbreviation for *total sugars as invert*.

Tunnel cap A contacting device used occasionally on *distillation plates*. It is essentially an elongated *bubble cap*, consisting of a chimney portion and a domed cover that deflects the *vapors* rising through the chimney, causing them to pass down through the liquid layer on the *plate.* Tunnel caps may be made from interlocking metal channels to form an integral part of the plate.

U

Upgrading A term used loosely to mean the *dehydration* of *hydrous ethanol*.

Ultrafiltration A process for the separation of colloidal or very fine solid materials, or large dissolved *molecule*s, by filtration through microporous or semi-permeable membranes. The process may be used for removal of *protein* from cheese *whey* prior to *fermentation*.

United States Department of Agriculture (USDA) A federal government department with the mission to improve and maintain farm income, to develop and expand markets for agricultural products, etc.

Urethane See *Ethyl carbamate*.

USDA Abbreviation for *United States Department of Agriculture*.

US gallon A measure of 231 cubic inches liquid at 60°F. It is the equivalent of 3.785 *liters* in the metric system or 5/6 of an *Imperial gallon*.

V

Vacuum distillation A process that takes advantage of the fact that liquids boil at lower temperature under reduced pressure. Thus, *distillation* under vacuum may be used for substances that otherwise have a relatively high *boiling point* such as *ethylene glycol* (which may be used in the *dehydration* of *ethanol*). The process may also be used in the distillation of *ethanol*-water mixtures, as the *azeotrope* is formed at a higher *proof* under lower pressures. Vacuum distillation may be used in the production of *rum* or *ethanol* from molasses to reduce the incidence of *scaling* in the *column* that occurs at high temperatures.

Vacuum fermentation A process of operating a *fermentation* under vacuum, so that the *ethanol* or other product is *vaporized* and removed as it is formed to avoid its concentration becoming inhibitory to the *yeast*. In a patented variation known as the Vacuferm process, instead of maintaining the entire *fermentor* under vacuum, the fermenting *beer* is circulated through a vacuum chamber to flash off the *ethanol* before returning the beer to the fermentor.

Vapor A dispersion in air of *molecule*s of a substance that is liquid or solid in its normal state at standard temperature and pressure. An example is water vapor or steam.

Vaporization (or Volatilization) The conversion of a chemical substance from a liquid or solid state to a *vapor* or gaseous state by the application of heat, by the reduction of pressure or by a combination of these processes.

Vapor pressure The saturation pressure exerted by *vapors* when in equilibrium with their liquid or solid forms.

Vent condenser The final *condenser* in a series of two or more connected to the *overhead vapor line* of a *distillation column*. As the final condenser, it is the one from which non-condensed (or non-condensable) gases and *vapors* are vented to the atmosphere or to a *scrubber* system.

Vinasse The term sometimes applied to the *stillage* of *molasses*, grape juice or other liquid *ethanol feedstocks*.

Vodka Currently defined by the *BATF* as '*neutral spirit* so distilled, or so treated after *distillation* with charcoal or other materials as to be without distinctive character, aroma, taste or color'. It should be noted that the charcoal treatment is now optional, whereas in earlier *BATF* regulations it was mandatory and the minimum time of treatment and amounts of fresh charcoal to be used were specified.

Volatile Adjective to describe a solid or liquid that readily converts to the *vapor* state.

Volatility The tendency of a solid or liquid to pass into the *vapor* state at a given temperature. With automotive fuels, the volatility is determined by measuring the *Reid vapor pressure (Rvp)*.

Volatilization See *Vaporization*.

W

Wash A British synonym for distillers *beer*.

Wet milling A process in which corn is first steeped (or soaked) in water containing sulfur dioxide. This softens the kernels and loosens the hulls. (The liquid when separated is known as *corn steep liquor*). The grain is then degermed by abrasion and liquid separation. Oil is extracted from the germ, while the remainder of the kernel is ground in impact fiber mills and passed through stationary screens to separate the *starch* and gluten from the fibrous portion. The heavier *starch* is then separated from the gluten by use of *centrifuges*. The *starch* portion may then be processed into commercial *starch*, or it may be used as a *feedstock* for production of *ethanol* or *HFCS*.

Weeping The condition when droplets of liquid fall through the holes of a *sieve plate* in a *distillation column*. It may be caused by a) steam flow that is too low, or b) too low a liquid flow to maintain a level on the plate, or c) having a tilted plate such that liquid depth is uneven.

Whey The serum or watery part of milk separated from the curd in the process of making cheese. It contains *lactose* and may be used as a *feedstock* for *ethanol* production.

Whisky Defined by the *BATF* as 'an alcoholic *distillate* from a *mash* of grain, produced at less than 190° *proof*, in such as manner that the distillate possesses the taste, aroma and characteristics generally attributed to whisky'. With the exception of *corn whisky*, it should be stored in oak barrels.

Whole stillage The entire stillage emerging from a *distillation* unit before any removal of solids by screening or *centrifuging*.

Wine gallon A *US gallon* of liquid measure as distinct from a *proof gallon*.

Wood alcohol See *methanol*.

Wort A brewery term used occasionally in distilleries. It refers to an unfermented *mash*, particularly if produced from a liquid *feedstock* or if the solids have been removed to yield a relatively clear, free-flowing liquid.

X

Xylene One of a group of three isomeric, aromatic *hydrocarbons* having a formula of $C_6H_4(CH_3)_2$. Commercial xylene is normally a mixture of the o- m- and p- *isomers* with a *boiling point* range of 137-145°C. It is a clear, flammable liquid. Derived from catalytic reforming of napthas or the distillation of coal tar, it may be used as an *octane enhancer* in *gasoline*.

Xylose A *pentose* (or 5-carbon sugar) derived from the *hydrolysis* of *hemicellulose*. Chemical formula $C_5H_{10}O_5$. It is not fermented by normal strains of distillers *yeast*s.

Y

Yeast Any of certain unicellular fungi, generally members of the class Ascomycetaceae (a few are members of the class Basidiomycetaceae). Many yeasts are capable of producing *ethanol* and *carbon dioxide* by *fermentation* of sugars. Yeasts are composed of approximately 35% *protein* and are a rich nutritional source of B vitamins.

Yeast autolysis The disintegration of *yeast* cells by the action of their own *enzymes*.

Yeast cream *Yeast* concentrated by centrifuging or decanting from the contents of a propagator or fermentor to be used as *inoculum* in another *fermentor*.

Yeast propagator (or pre-fermentor) A tank used for the propagation or development of a *yeast* culture prior to transfer to a *fermentor*. It is normally fitted with aeration, agitation and cooling devices and is designed for ease of cleaning and sterilization.

Yeast recycle See *cell recycle*.

Yeast strain A pure culture of *yeast* derived from a single isolation. Strains may be specially selected for certain characteristics such as the ability to efficiently produce and tolerate high temperatures or high levels of *ethanol* (see Thermosacc™).

Z

Zeolite See *Molecular sieve*.

Zymomonas A genus of the Pseudomonadaceae family of *bacteria* which are characterized by being *Gram-negative* and non-spore-forming. The genus Zymomonas is distinguished by its *fermentation* of *sugar* to *ethanol*. The principal species being examined commercially for *fuel ethanol* production is *Zymomonas mobilis*. It is, however, considered an undesirable contaminant in *beverage alcohol fermentation*s in that it tends to produce hydrogen sulfide from sulfur compounds in the *mash*, particularly that derived from *molasses*.

Index

Acetobacter (see Bacterial contaminants) 235
Acidic cleaners 306
Active dry yeast 96-97, 111, 121-122, 141
Acrolein 103, 206
Agave (see also Tequila)
  Beverages from 223-224
  Blue agave cultivation 226-228
Alkali 305
Alpha amylase
  Bacillus sources 29
  Calcium 19
  Cooking systems 16
  Hydrolysis of starch 14, 29-32
  In malt 15, 29-31
  pH ranges 16, 18-19
  Temperature ranges 18-19, 20
Allcoholase™ 12, 17, 18, 21
Allpen™ 126
Allprotease™ 117
Allzyme™ SSF
Ammonia 115
Amy alcohol (see fusel oils)
Amyloglucosidase (see glucoamylase)
Amylose
  Content of cereals 13-14, 27-28
  Structure 14, 26-27
Amylopectin
  Content of cereals 13-14, 27-28
  Cassava 62
  Structure 15, 26-27
Anhydrous ethanol 335-336
Antimicrobials
  Against infection 126, 292-296
  Residues in DDGS 126, 293
  Continuous culture 142
Araban 46
Arabanase 46
Arabinose
  Metabolism by fungi and bacteria 48-50
AYF™ 82
Azeotrope 321-323, 337

Backset
  Acidification with 21
  Amounts added 16
  Composition 16, 346
  Contaminants 16, 284
  FAN in, 347
  NIR analysis of 165
Bacterial contaminants 288
  Acetobacter 235, 287-288, 290
  Agave fermentation 235
  Continuous fermentation 140
  Gluconobacter 287-288
  Lactic acid bacteria 123-124, 206-208, 288-292
    Inhibition of yeast 110, 290-292
    Loss of spirit due to 102, 110, 126, 208
    Off flavors 206
    Practical controls 126, 292-296
    Saccharification and 21
    Rum 249
  Water recycling and 343-350
Barley (see also Malt) 10, 12, 200, 217
Barrelling 211, 217, 240-241, 251, 284-285
Batch fermentation 130, 135
Beerstone 131-132, 304
Beta amylase 15, 29-31
Biofilm 304
Biomass to ethanol (see lignocellulosics)
Biomethanator 343-344, 350-351
Biorefinery 33, 389-398
  Bacterial biocatalysts 397-398
  Biotransformation of DDGs 394
Biostill fermentor 138-139
Bleach 306, 315
BOD
  Whey 68, 71
  Wastewater 374
Botanicals (see flavored beverages)
Bourbon
  History of 276-277
  Production of 278-285
  Fermentation 281-282
  Distillation 278, 282-283
Brettanomyces (see wild yeast)
Brix defined 78

CakeGuard™ 382-383
Calcium
  Enzymes and 11
  Yeast and 106-108
Canadian whisky 260-261
Candida
  C. utilis 176

  C. tropicalis 177
  C. famata 177
  C. lipolytica 178
  C. guilliermondii 178
  C. kefyr 179
  C. boidinii 180
Carbon dioxide 122, 131, 140, 391
  Scrubber CIP 314
  Scrubber water composition 348-349
Carotenoids 172
Cassava
  Composition of chips 59
  Fermentation of 62-63
  World production 60
  Ethanol from 60-64
Caustic 309, 314, 316
Cellulose
  Content in biomass sources 42-43
  Structure 26-27, 44
Centrifuges 366-368
Chelating agents 305-306
Chlorine dioxide (see Iotech™) 131, 250, 307-308
CIP systems 131-132, 301
  Cycle examples 314-316
  NIR and 162
Citric acid Cycle (see TCA)
Clean Air Act 2, 72
Cleaning and sanitizing 299-318
  Cleaning loops 312-313
  Four Ts 299
  Evaluation 317-318
  Temperature and 302-303
  Sanitary design 309-312
  Soil types 304
  Solutions 304-309
Clostridium saccharobutyricum 249
Cocktails 268
COD
  Whey 68
COP 301
Coffey still 193, 208, 255, 259-260
Congeners (see fusel oils)
Continuous fermentation 132-133, 135-143
  Cleaning 301
  Contamination in 136, 139-143
  Steady state 137
Conversions
  Mole fraction to % alcohol 258
Cooking 203, 344
  Agave 230-231
  Batch 15-16, 19
  Cassava 62
  Continuous 17-19, 203-204
  Jet cooker 18
  Relative heat requirements 18
Cooling methods 125, 131
Cooperage (see Barrelling)
Copper
  Yeast and 106-108
  Lignocellulosics and 47
Co-products (see also DDGS, Biorefinery) 363-365, 393
Cordials 268
Corn
  Grind size 9-11
  Gelatinization of 12
  Yield from 10
  Sieve analysis 10, 38
  Starch in 27-28
Crabtree effect 96
Cream yeast 96-97
Cryptococcus laurentii 180
Cylindroconical fermentor 130-131

DDG 392-394
DDGS 5, 33-34
  Antibiotics in 126, 293, 384
  Composition 381
  Dairy and beef cattle use 380-381
  Mycotoxins and 34-35, 128, 384
  NIR analysis of 156, 164-165
  Nutritive value for animals 378
  Pigs and poultry 383-384
  Production in NA 377
  Phosphorus availability 379-380, 383-384
  Sanitation 39
  Storage 375
DE 11, 20-21, 29
Debaromyces hansenii 177-178
Dekkera (see wild yeast)
Denatured ethanol 355
Dextran 78
Dextranase
Dextrins 11, 14, 29
  Limit dextrins 14, 29
  HPLC and 15
Disaccharides 25
Distillation
  Azeotropic distillation 321-323, 337
  Beverages 83, 239, 250-251, 255-266, 282-284
  Barbet head 262-263
  Contact devices 324, 330-331
  Continuous stills 210-211, 239, 259
  Demethylizer 262-263
  Energy use 264-265, 324-326, 331-333
  Extractive distillation 262
  Fundamentals explained 319-324
  Fusel oil removal 263-264, 266, 334
  Heat recovery 265
  Origins 194
  Packed column 264
  Pinch point 262
  Pot stills 204, 208-210, 255-257
  Pressure distillation 264-265
  Reboilers 264, 331
  Rectifier 250, 261-262, 319

  Thermocompressors 265, 331
  Tower sizing 327-330
  Vacuum distillation 265
Distillers bushel 10
Dryers 371-374
Dryhouse
  Design and equipment 365-375
  Energy use 360
  NIR applications 164-165

E85 5
Ediesel 5
Effluent treatment and disposal 374
  Molasses 83-84
  Tequila 239-240
  Whey processing 71-72
  Whisky 213
Emissions 374-375
Energy use
  Distribution in plant 359-361
  Factors affecting 357-359
  Thermal and electrical ratios 356-357
Esters 109-110, 213, 238, 249
Esterase 46
Ethanolgenic microbes 47
Ethyl acetate (see esters)
Ethyl caproate (see esters)
Evaporators 369-371
  Condensate 348, 374

FAN 113-114, 346
Fermentation (see Batch, Continuous)
  Monitoring with NIR 159
Flavor compounds
  Distillation and 255-266
  Lactic bacteria and 249
  NCY 172
  Produced by yeast 104-105, 213-220
Flavored beverages 258-266
  Artificial vs natural 269
  Botanicals 258-259, 269
  Color 271
  Extracts 269
  Flavor perception 267
  Tax benefits 270, 271
  Types of 268-269
  Vodka 255
Flocculation 105
Fuel ethanol industry 1-7
  Asia 6
  Australia 5, 6
  Brazil 5-6
  China 5-6
  EU 6, 72
  History 1-2
  India 5
  Thailand 5
  US 2-5, 41, 72

Fungi 396-397
Fusel oils 108-110, 208, 212, 235-238, 262-263
Fructose
Fruit 262

Galactan 46
Galactanase 46
Gasohol 1
Gelatinization 11-12, 28
  Cassava 62
Glucoamylase 20, 29-31
  Activity 14-15
  Conventional vs Rhizozyme™ 20
  pH 16, 110
  Source organisms 21, 29-30

Gin 255, 258-259
Glucose
  Structure 25-26
Glucan
  In lignocellulosics 46
  In yeast cell wall 87-88
Gluconobacter (see Bacterial contaminants)
Glycerol 102, 206, 391
Glycolysis 23-24, 92
Glycogen 26
  In yeast 87-88, 112
  Structure 112
GMPs 33-34
Grain handling 33-38, 280
  Contaminants 34-35
  Moisture content 35
  NIR analysis 158-159
  Sampling 35
  Sanitation 37-38
Grain whisky 203-204
Grind 9-11, 61, 202
Guiacol 201
Guy-Lussac equation 93

Hansenula polymorpha 182
Hayflick Limit 88
Hammer mill 9-10
Heat
  Control equipment 125
  Production by yeast 122-123
Hemicellulose
  Composition 43
  Structure 44
Hemicellulase 47
High gravity fermentation 117
Higher alcohols (see fusel oils)
High T™ 17, 18
HPLC vs NIR 159-162
Hydrofining 262
Hydroheater 18

Industrial ethanol 333-335

Infection (see Bacterial contaminants)
Inulin (see also Tequila) 230-231
Invert sugars 77
Iotech™ 314
Irish whiskey 194-195, 255-256
  vs Scotch 197
  Production 197-213
Iron 106-107
Isoamyl acetate (see esters)
Isoamyl alcohol (see fusel oils)
Isobutyl acetate (see esters)
Iodophors 131, 302, 309

Jet cooker 18

Killer yeast 98-99
Kluyveromyces
  K. lactis 179
  K. marxianus 71, 179
Kreb's Cycle (see TCA)

Lactic acid (see also Bacterial contaminants)
  Inhibition of yeast 110, 290-292
  In steep water 345
  Measurement with NIR 162-163, 165
Lactoside™ 63, 126, 294-296
Lactose 25, 69
  Structure 71
  Hydrolysis of 70
Lag phase 94
Leuconostoc, 206
  Molasses contaminant 83
Lignin 43
  Ligninases 47
Lignocellulosics
  Composition 41-43
  Enzymes for 46-47
  Microbial fermentation of 47-52
  Processing 44-46
  Sources 41
Liquefaction 11-21, 62
Log phase 96

Magnesium 106-108
Maillard reactions 121
Malt 25, 199-201, 202, 279
  Enzymes 11, 15, 18, 117
Malternatives 268-269
Maltose 91, 205
Maltotriose, 205
Manganese 106-108
Mannan
  In lignocellulosics 46
  In yeast cell wall 87-88
Mash tun 202
Maturation 211-213, 218, 260, 240, 251-252, 284-285
Methanol 237, 262
Methylene blue stain 101
Milo (see sorghum)
Milling 9-11, 37-38
  Cassava 61
  Agave 231-232
Molasses (see also Rum) 23, 75-84
  Cane vs beet 76
  Composition of 76-78
  Dextrans 78
  Dilution of 79-80
  Fermentation 80-83
  Handling 79-80
  Types of 75-76
  World production 75
Molecular sieve 337-341
Monosaccharides 25
MTBE 2, 4-5, 356
Mycotoxins 34-35, 128, 384

Neutral spirit 10, 259-263
NIR 9, 145-169
  Calibration 153-158
  Dryhouse applications 164-165
  Ethanol 159-162, 166-169
  Lactic acid 162
  Grain quality 158-159
  Measuring techniques 150-152
  Spectrometer types 147-149
  Starch analysis 163-164
  Sugars 161-162
  Troubleshooting fermentors 163
Non-conventional yeast (NCY) 171- 190, 396
  Taxonomy 171-172
  Products of 172
  Physiology of 175-177
n-propanol (see fusel oils)

Oats 200
Oligosaccharides 25
Organoleptic compounds
  Scotch 213-220
  Tequila 235-238
Oxygenates 356

Peracetic acid 308
Pichia
  P. angusta 182
  P. stipitis 48-49, 182
  P. guilliermondii 178-179
  P. pastoris 181
Phaffia rhodozyma 181-182
Phenethyl alcohol (see fusel oils)
Phytic acid 129
Polysaccharides 25
Potassium 106-107
Potatoes 262
Pullulanase 30, 31
Pressed yeast 96-97, 121-122
Protease 117

Quaternary ammonium compounds 308-309

Reducing sugars 77
Reformulated gasoline 2
Rice 12
Roller mill 10-11, 202-203
Rum (see also Molasses)
  Types of 247-248
  Feedstocks 248-249
  Heavy rum 249
  Lactic bacteria 249
  Light rum 249-250
  Distillation 250-251
Rhizozyme™ 9, 10, 12, 17, 18, 21, 117, 126, 128
  Agave wort 235
  Cassava 62-63
  Energy use and 358
  vs conventional gluc 20, 124-125
Rye 10, 12, 355
  Whisky 275, 279, 282

Saccharification 11, 14-16, 20
Saccharomyces cerevisiae in ethanol production
  Acid washing 132
  Aerobic vs anaerobic metabolism 92-94
  Amino acid uptake 115
  Budding 87, 101-102, 124
  Cell features 86-88
  Cell growth phases 94, 127
  Cell wall structure 87-88
  Composition 88
  Conditioning yeast 97, 127-128, 234, 280-281
  Counting 100
  Energy 94-95
  Ethanol tolerance 117, 122, 127
  Ester formation 109-110
  Flavor compounds 104-105, 109
  Foods 117
  Fusel/higher alcohols 108-109
  Glycolysis in 92-94
  Glucose tolerance 125-125
  Growth requirements 89-91
  Magnesium 107-108
  Metabolic engineering 395
  Mineral requirements 106-107
  Molasses and nutrition of 82
  Mutations 113
  Mycotoxins 128
  Nitrogen and FAN 113-117
  Oxygen use by 96, 110
  pH optima 102
  Phosphorus 107
  Pitching 100
  Products of fermentation 102
  Propagation of 96-97
  Sucrose hydrolysis by 90
  Sugar uptake 90-91, 93, 205
  Sulfur metabolism 104, 107
  Size of 86
  Sterol needs 110-111
  Strain selection 121-122
  Stress factors 117, 122-130, 349-350
  Taxonomy 85
  Temperature tolerance 99-100, 122
  Viability 100-102
  Vitality 100
  Vitamin needs 105
  Xylose fermenting 48-52, 395-396
Saccharomyces pombe 182
Sanitizing 301-302, 307-309
Scale 126, 315
Scale-Ban™ 126
Scale-Bite™ 314
Schizosacharomyces pombe 182
Scotch whisky 15, 255
  Authenticating malts 219-220
  Blending 213
  Defined 193-194
  Maturation 211-213
  Organoleptic compounds 213-220
  Production 199-213
  Regions 196-
  vs Irish 197
  Yeast 204
Sieve analysis 10, 38, 279
Simultaneous saccharification and fermentation 20, 29, 48, 62, 140
Soils 304
Sodium
  Yeast and 108, 351-352
Sorghum 12, 355
  DDGS 379
Spent grains (see also DDGS) 202
Starch 26-32 (see amylose, amylopectin)
  Hydrolysis of 12-15, 23-32
Steep water composition 345
Sterols 110, 111-112
Stillage
  Thin stillage 346-347
  Whole stillage 363-365
  Heat recovery 366
Stuck fermentation 113, 206
Sugarbeets (see molasses) 75
Surfactants 306
Succinic acid 103, 391
Sucrase 23
Sucrose 23
  Structure 77
Sugar cane (see molasses)
Sulfur
  Metabolism by yeast 104
Surface culture enzyme (see Rhizozyme™)
Superstart™ 121

TCA cycle 88, 103-104
Tequila 223-244

  Contamination 235-236
  Cooking 230-232
  Fermentation 232-236
  Maturation 240-241
  Organoleptic characteristics 235
  Origin and history 224-225
  Processing agave 229-230
  Production stats 241-42
  Yeast 232-236
Thermal oxidizers 375
Thermosacc™ 17, 18, 82, 100, 121, 128, 233
Thin stillage (see also Stillage, Evaporators) 346-347
Torula utilis 176
Total sugars as invert 78
Trehalose 112-113

Urea 115
U-tube cooking system 17-19

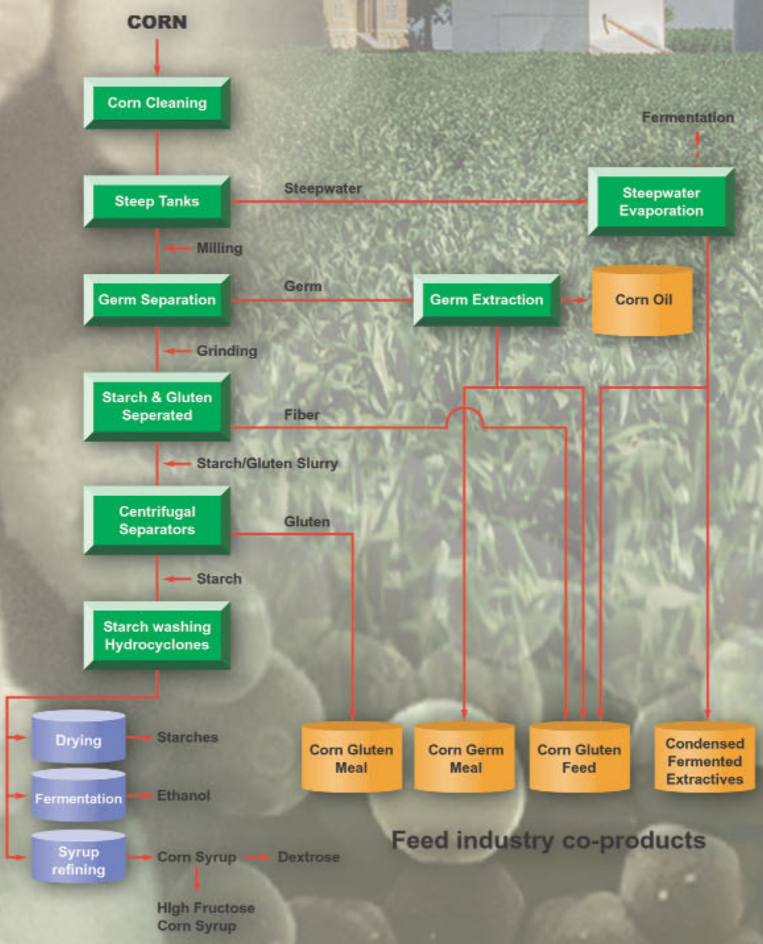
Viscosity 11-12, 17, 18-19
  Molasses 79
Vitamins in yeast metabolism 105-106
  NCY 172
Vodka 259, 275
  Flavored 267

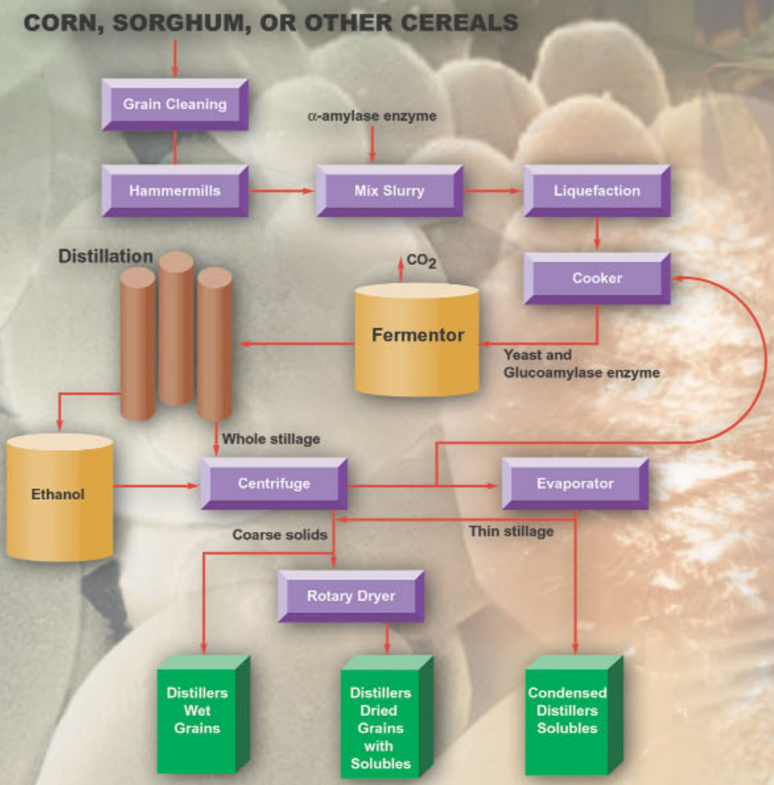
Water reuse 343
  Effect on yeast 349-353
Wet grains 382-383
Wheat 12
Whey
  Composition 65, 70
  Ethanol production from 70-72
  Pollution and 65, 68
  Production of 65, 69
  Uses of 68-70
Whisky (see Scotch, Irish, Canadian, Bourbon)
Wild yeast 98

Xylan 43
Xylose
  Metabolism by fungi and bacteria 48-50
Xlyanase 46

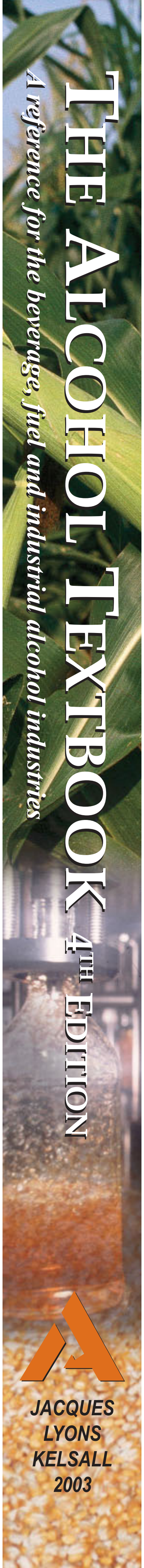
Yarrow lipolytica 178
Yeast (see also *Saccharomyces cerevisiae* other yeast species, wild yeast, killer yeast, nonconventional yeast)

Zeolites 337
Zero effluent design 353
Zinc 106-108 (see *S. cerevisiae* mineral needs)
Zygosaccharomyces rouxii 180
Zymomonas mobilis 48, 397
  Molasses contaminant 83


CORN
Corn Cleaning
Steep Tanks
Steepwater
Steepwater Evaporation
Fermentation
Milling
Germ Separation
Germ
Germ Extraction
Corn Oil
Grinding
Starch & Gluten Seperated
Fiber
Starch/Gluten Slurry
Centrifugal Separators
Gluten
Starch
Starch washing Hydrocyclones
Drying
Starches
Fermentation
Ethanol
Syrup refining
Corn Syrup
Dextrose
High Fructose Corn Syrup
Corn Gluten Meal
Corn Germ Meal
Corn Gluten Feed
Condensed Fermented Extractives
Feed industry co-products


CORN, SORGHUM, OR OTHER CEREALS
Grain Cleaning
α-amylase enzyme
Hammermills
Mix Slurry
Liquefaction
Cooker
Distillation
CO2
Fermentor
Yeast and Glucoamylase enzyme
Whole stillage
Ethanol
Centrifuge
Evaporator
Coarse solids
Thin stillage
Rotary Dryer
Distillers Wet Grains
Distillers Dried Grains with Solubles
Condensed Distillers Solubles

NOTTINGHAM
University Press



THE ALCOHOL TEXTBOOK 4TH EDITION

A reference for the beverage, fuel and industrial alcohol industries

JACQUES
LYONS
KELSALL
2003